

**PCT**WORLD INTELLECTUAL  
PROPERTY ORGANIZATION

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>A61K 31/52, C07D 473/00, C07H 19/167, 19/173</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/03125</b>
			<b>(43) International Publication Date:</b> 8 February 1996 (08.02.96)
<b>(21) International Application Number:</b> PCT/US95/10008		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).	
<b>(22) International Filing Date:</b> 25 July 1995 (25.07.95)			
<b>(30) Priority Data:</b> 08/280,719 25 July 1994 (25.07.94) US 08/488,976 8 June 1995 (08.06.95) US 08/492,929 20 July 1995 (20.07.95) US			
<b>(71)(72) Applicants and Inventors:</b> GLASKY, Alvin, J. [US/US]; 12231 Pevero, Tustin, CA 92680 (US). RATHBONE, Michel [CA/CA]; 40 Spadina Avenue, Hamilton, Ontario L8M 2X1 (CA).		<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(74) Agents:</b> MACLEAN, Kurt, A. et al.; Poms, Smith, Lande & Rose, Suite 3800, 2029 Century Park East, Los Angeles, CA 90067 (US).			
<b>(54) Title:</b> CARBON MONOXIDE DEPENDENT GUANYLYL CYCLASE MODIFIERS			
<b>(57) Abstract</b> <p>Disclosed herein are methods and associated compositions and medicaments directed generally to the control of cellular and neural activity and for selectively and controllably inducing the <i>in vivo</i> genetic expression of one or more naturally occurring genetically encoded molecules in mammals. More particularly, the present invention selectively activates or derepresses genes encoding for specific naturally occurring molecules such as proteins or neurotrophic factors and induces the endogenous production of such naturally occurring compounds through the administration of carbon monoxide dependent guanylyl cyclase modulating purine derivatives. The methods of the present invention may be used to affect a variety of cellular and neurological functions and activities and to therapeutically or prophylactically treat a wide variety of neurodegenerative, neurological, cellular, and physiological disorders.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

**CARBON MONOXIDE DEPENDENT GUANYLYL CYCLASE**  
**MODIFIERS**

**FIELD OF THE INVENTION**

The present invention relates in general to the control of cellular and neural activity and to the treatment of cellular and neural disorders. More particularly, the present invention is directed to methods and associated compositions and medicaments for the modification of mammalian cellular and neural activity through the administration of carbon monoxide dependent guanylyl cyclase modulating purine derivatives which selectively and controllably induce the *in vivo* genetic expression of naturally occurring genetically encoded molecules including neurotrophic factors. The methods, compositions, and medicaments of the present invention may be used to affect a variety of cellular and neurological activities and to therapeutically or prophylactically treat a wide variety of physiological, neurodegenerative, and neurological disorders.

**BACKGROUND OF THE INVENTION**

The evolution of the central nervous system in mammals was a natural response to an increasingly complex environment requiring solutions to difficult problems. The resulting structure is an intricate biochemical matrix that is precisely controlled and attenuated through an elaborate system of chemically modulated regulatory pathways. Through an elaborate series of highly specific

-2-

chemical reactions, these pathways oversee and direct every structural and operational aspect of the central nervous system and, through it, the organism itself. Normally the complex interplay of the various control systems  
5 cooperates to produce a highly efficient, versatile central nervous system managed by the brain. Unfortunately, when the biochemical matrix of the central nervous system is damaged, either through age, disease or other reasons, the normal regulatory pathways may be incapable of effectively compensating for the loss. In such cases it would  
10 be highly desirable to modify or supplement the neural mechanisms to prevent or compensate for such disorders. That is the focus of the present invention.

More specifically, the mammalian brain is composed of  
15 approximately ten billion nerve cells or "neurons" surrounded by a even greater number of support cells known as neuroglia or astrocyte cells. Neurons, like other cells of the body, are composed of a nucleus, a cytoplasm and a surrounding cell membrane. However, unlike other cells,  
20 neurons also possess unique, fiberlike extensions allowing each individual nerve cell to be networked with literally thousands of other nerve cells to establish a neural infrastructure or network. Communication within this intricate network provides the basis for all mental processes  
25 undertaken by an organism.

In each nerve cell, incoming signals are received by neural extensions known as "dendrites" which may number several thousand per nerve cell. Similarly, neural information is projected along nerve cell "axons" which may  
30 branch into as many as 10,000 different nerve endings. Together, these nerve cell axons and dendrites are generally termed "neurites" through which each individual neuron can form a multitude of connections with other neurons. As a result, the number of possible neural connections in  
35 a healthy brain is in the trillions, giving rise to tremendous mental capacity. Conversely, when the connections

-3-

within the neural network break down as nerve cells die or degenerate due to age, disease, oxidative stress, or direct physical insult, the mental capacity of the organism can be severely compromised.

5       The connection of the individual axons with the dendrites or cell bodies of other neurons takes place at junctions or sites known as "synapses." It is at the synapse that the individual neurons communicate with each other through the flow of chemical messengers across the  
10 synaptic junction. The majority of these chemical messengers, or "neurotransmitters," are small peptides, catecholamines or amino acids. When the appropriate stimulus is received by a neural axon connection, the neurotransmitters diffuse across the synapse to the adjacent neuron,  
15 thereby conveying the stimulus to the next neuron along the neural network. Based upon the complexity of the information transferred between the nerve cells, it is currently believed that between 50 and 100 distinct neurotransmitters are used to transmit signals in the  
20 mammalian brain.

Quite recently, it was discovered that nitric oxide (NO) and carbon monoxide (CO) may function as neurotransmitters. These gaseous molecules appear to participate in a number of neuronal regulatory pathways affecting cell  
25 growth and interactions. In the brain, as well as in other parts of the body, CO is produced by the enzyme "heme oxygenase II" (HO). Whether produced from the HO enzyme or from other sources, it is believed that when CO diffuses into a neuron it induces a rise in a secondary  
30 transmitter molecule known as "cyclic guanosine monophosphate" (cGMP), by modulating an enzyme known as "guanylate cyclase" or "guanylyl" cyclase. Thus, CO acts as a signaling molecule in the guanylyl cyclase regulatory pathway. The resultant increase in cGMP levels appears to  
35 modify several neurotropic factors as well as other neuronal factors which may induce, promote or modify a variety

-4-

of cellular functions including cell growth, protection, and intercellular communication.

Neurotrophic factors are molecules that exert a variety of actions stimulating both the development and differentiation of neurons and the maintenance of cellular integrity and are required for the survival and development of neurons throughout the organism's life cycle. Generally, neurotrophic factors may be divided into two broad classes: neurotrophins and pleiotrophins. Pleiotrophins differ from the neurotrophins in that they lack a molecular signal sequence characteristic of molecules that are secreted from cells and they also affect many types of cells including neurons. Two effects of neurotrophic factors are particularly important: (i) the prevention of neuronal death and (ii) the stimulation of the outgrowth of neurites (either nascent axons or dendrites). In addition, it appears that CO-induced neurotrophic factors may reduce the membrane potential of nerve cells making it easier for the neurons to receive and transmit signals.

Many of today's researchers believe that memory is associated with the modification of synaptic activity, wherein the synaptic connections between particular groups of brain neurons become strengthened or facilitated after repeated activation. As a result, these modified connections activate much easier. This type of facilitation is believed to occur throughout the brain but may be particularly prominent in the hippocampus, a brain region which is crucial for memory. The stimulation of neuronal pathways within the hippocampus can produce enhanced synaptic transmission through these pathways for many days following the original stimulation. This process is known as long term potentiation (LTP).

More particularly, long term potentiation is a form of activity-dependent synaptic electrical activity that is exhibited by many neuronal pathways. In this state, generally accepted as a type of cellular memory, nerve

-5-

cells are more responsive to stimulation. Accordingly, it is widely believed that LTP provides an excellent model for understanding the cellular and molecular basis of synaptic plasticity of the type that underlies learning and  
5 memory in vertebrates, including man.

NO and CO are currently the leading candidates for messenger substances that facilitate LTP because inhibitors of these compounds retard the induction of potentiation. The ability to modify neural activity and  
10 to increase the ease of LTP using these or other signal transducers could potentially increase learning rates and cognitive powers, possibly compensating for decreased mental acuity. Prior to the present invention, there were no known methods or agents which could operate on the  
15 cellular level *in vivo* to reliably modify cellular and neural regulatory pathways so as to facilitate the LTP of neurons.

In contrast to the enhanced mental capacity provided by long term potentiation, mental functions may be impeded  
20 to varying degrees when the neuronal network is disrupted through the death or dysfunction of constituent nerve cells. While the decline in mental abilities is directly related to the disruption of the neural network, it is important to remember that the disruption is occurring on  
25 an individual cellular level. At this level the deleterious effects associated with neuronal disruption may be brought about by any one of a number of factors including neurodegenerative diseases and disorders, heart attack, stroke, aging, trauma, and exposure to harmful chemical or  
30 environmental agents.

Among the known neurological diseases which adversely impact neuronal function are Alzheimer's disease and related disorders, Parkinson's disease, motor neuropathic diseases such as Amyotrophic Lateral Sclerosis, cerebral  
35 palsy, multiple sclerosis, and Huntington's disease. Similar problems may be brought about by loss of neuronal

-6-

connectivity due to normal aging or through damage to neurons from stroke, heart attack, or other circulatory complications. Direct physical trauma or environmental factors including chemical agents, heavy metals and the  
5 like may also provoke neuronal or cellular distress, dysfunction, or death.

Accumulated cellular damage due to oxidative free radicals is believed to be one of the critical factors in a variety of cellular and neurodegenerative diseases  
10 including Amyotrophic Lateral Sclerosis, Parkinson's disease, Alzheimer's disease, cancer, and aging. Most cells possess a variety of protective mechanisms that guard against cytotoxic free radicals. For example, high levels of glutathione may protect against free radical  
15 oxidation. Neurons are deficient in this antioxidant source.

Whatever the cause of the neural disorder or dysfunction, the general inability of damaged nerve cells to undergo substantial regrowth or regeneration under natural  
20 conditions has led to the proposal that neurotrophic factors be administered to nerve cells in order to help restore neuronal function by stimulating nerve growth and function. Similarly, stimulating neuritogenesis, or the growth of neurites, by administering neurotrophic factors  
25 may contribute to the ability of surviving neurons to form collateral connections and thereby restore neural function.

At present, prior art techniques and compounds have not been effective or practical to directly administer  
30 neurotrophic factors to a patient suffering from a neural disorder. In part, this is due to the complex molecular interaction of the neurotrophic factors themselves and to the synergistic regulation of neural cell growth and neuritogenesis. Neurotrophic factors are the result of a long  
35 chemical cascade which is exquisitely regulated on the molecular level by an intricate series of transmitters and



-7-

receptors. Accordingly, neuronal cells are influenced by a concert of different neurotrophic factors, each contributing to different aspects of neuronal development at different times. Neurotrophic factors are, effectively, the  
5 tail end of this cascade and thus are one of the most complex components of the regulatory pathway. As such, it was naive for prior art practitioners to assume that the unattenuated administration of single neurotrophic factors at random times (from the cells viewpoint) could substantially improve cell activity or regeneration. In contrast, modification of the regulatory pathway earlier in the cascade could allow the proper growth factors to be produced in the correct relative amounts and introduced into the complex cellular environment at the appropriate  
10 time.  
15

Other practical considerations also preclude the prior art use of neurotrophic factors to stimulate the regeneration of the neuronal network. Neurotrophic factors (including neurotrophins and pleiotrophins) are large  
20 proteins and, as such, are not amenable to normal routes of medical administration. For example, these proteins cannot be delivered to a patient or subject orally as the patient's digestive system would digest them before they reached the target neural site. Moreover, due to their  
25 relatively large size, the proteins cannot cross the blood brain barrier and access the most important neurological site in the body. Alternatively, the direct injection of neurotrophic factors into the brain or cerebrospinal fluid crudely overcomes this difficulty but is fraught with  
30 technical problems of its own which have thus far proven intractable. For example, direct infusion of known neurotrophins into the brain has proven impractical as it requires administration over a period of years to provide therapeutic concentrations. Further, direct injection  
35 into the brain has been associated with dangerous swelling and inflammation of the nerve tissue after a very short

-8-

period of time. Thus, as theoretically desirable as the direct administration of neurotrophic factors to a patient may be, at the present time, it is unfeasible.

Accordingly, it is a general object of the present invention to provide methods and associated compositions and medicaments for effectively modifying mammalian cells, neurons, cellular activity, or neural activity to achieve a variety of beneficial results. These results include protection against oxidative stress and damage by free radicals and more generalized physiological responses such as reductions in mammalian blood pressure.

Thus, it is another object of the present invention to provide methods and associated compositions and medicaments for treating mammalian neurological diseases and cellular disorders.

It is yet another object of the present invention to provide methods and associated compositions and medicaments for inducing long term changes in the membrane potential of a mammalian neuron.

It is still another object of the present invention to provide methods and associated compositions and medicaments for inducing the *in vivo* physiological production and administration of genetically encoded molecules and neurotrophic factors within cells.

It is a further object of the present invention to provide methods and associated compositions and medicaments for enhancing the neurotogenic effects of neurotrophic factors in a physiological environment.

#### SUMMARY OF THE INVENTION

These and other objects are accomplished by the methods, compositions, and medicaments of the present invention which, in a broad aspect, provide for the selective inducement of the *in vivo* genetic expression and resultant production of naturally occurring genetically encoded molecules including neurotrophic factors, and for

-9-

the modification of cellular and neural activity through the treatment of mammalian cells and neurons with at least one carbon monoxide dependent, guanylyl cyclase modulating, purine derivative.

5       As will be appreciated by those skilled in the art, the *in vivo* activation or derepression of genetic expression and the exemplary modifications of cellular and neural activity brought about by the methods, compositions, and medicaments of the present invention may  
10 be expressed in a variety of forms or combinations thereof. For example, the treatment of a mammalian cell or neuron through the teachings of the present invention may result in the cell's direct self-administration of the *in vivo* expressed molecule(s) through the enhanced cellu-  
15 lar production of various naturally occurring genetically encoded compounds, such as proteins and neurotrophic factors, or in the stimulation of the activity of those compounds and their subsequent effect on naturally occurring cellular or neuronal metabolism, function,  
20 development, and survival. These subsequent effects can include protection from free radical oxidation and cellular destruction, stabilization of cell receptors against other factors, the endogenous production of carbon monoxide and antioxidant compounds, and even reductions in  
25 blood pressure via carbon monoxide activated cellular mechanisms. The methods and medicaments of the present invention may also stimulate the growth, development and survival of the cell or neuron directly without the deleterious effects of prior art factor methodology.  
30 Further, the present invention may be used to lower or change the membrane potential of the cell, increasing its plasticity and inducing long term potentiation.

Exemplary carbon monoxide dependent guanylyl cyclase modulating purine derivatives useful for practicing the  
35 present invention include guanosine, inosine pranobex and 4-[3-(1,6-dihydro-6-oxo-9-purin-9-yl)-1-oxopropyl]amino]

-10-

benzoic acid (AIT-082). Unlike prior art compounds, these compounds may be administered directly to a patient either orally or through injection or other conventional routes. These exemplary compounds are nontoxic and will cross the  
5 blood-brain barrier as well.

In a further, more specific aspect, the methods and compositions of the present invention may be used for the treatment or prophylactic prevention of neurological diseases and other cellular disorders, including those  
10 brought about by disease, oxidative stress, age, trauma or exposure to harmful chemical agents. By promoting the survival, growth and development of individual neurons and cells, the present invention facilitates the regeneration and development of the neural network and alleviates the  
15 manifestations of cellular and neural dysfunction.

Of course, those skilled in the art will appreciate that pharmaceutical compositions and medicaments may be formulated incorporating effective concentrations of the carbon monoxide dependent guanylyl cyclase modifying  
20 purine derivatives of the present invention along with pharmaceutically acceptable excipients and carriers. These pharmaceutical compositions may be administered orally, transdermally, topically or by injection. Moreover, as the active agents used in the methods of the  
25 present invention can cross the blood-brain barrier, they do not have to be injected or infused directly into the brain or central nervous system.

In yet another aspect, the methods and compositions of the present invention may be used to induce long term  
30 changes in the membrane potential of a mammalian neuron. These long term potentiation changes may lead to increased membrane plasticity with a corresponding enhancement of cellular memory. In turn, this enhanced cellular memory may elevate the mental capacity of the subject leading to  
35 faster learning and increased retention of material.

-11-

Other objects, features and advantages of the present invention will be apparent to those skilled in the art from a consideration of the following detailed description of preferred exemplary embodiments thereof taken in conjunction with the data expressed in the associated figures which will first be described briefly.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graphical representation of murine plasma concentration following administration of the purine derivative AIT-082 in accordance with the present invention;

Fig. 2 is a graphical representation of the effect of atropine, a cholinergic antagonist, on memory enhancement in mice by the purine derivative AIT-082;

Fig. 3 is a graphical representation of nerve growth factor mediated neurotogenic response in neuronal cells grown *in vitro* with various concentrations of the purine derivative AIT-082;

Figs. 4A, 4B and 4C are graphical comparisons of the effects of selective inhibitors and the purine derivative AIT-082 on nerve growth factor mediated neurotogenic response; Fig. 4A shows the neurotogenic response of cells grown in the presence of methemoglobin, a carbon monoxide scavenger; Fig. 4B shows the same response of cells grown in the presence of methylene blue, a guanylyl cyclase inhibitor Fig. 4C shows the response of cells grown in the presence of zinc protoporphyrin IX, a carbon monoxide scavenger;

Figs. 5A and 5B are graphical comparisons of nerve growth factor mediated neurotogenic response for cells grown in the presence of the purine derivative AIT-082 and various concentrations of nitric oxide inhibitors;

Fig. 6 is a graphical comparison of cyclic GMP production in neuronal cells grown in culture with the purine derivative AIT-082 and without AIT-082;

-12-

Fig. 7 is a graphical representation of the effects of different doses of the purine derivative AIT-082 on learning as measured in Swiss Webster mice using a win-shift memory test;

5 Fig. 8 is a graphical comparison of the duration of action of the purine derivative AIT-082 measured over time for single doses of 60 mg/kg and 30 mg/kg;

Fig. 9 is a graphical comparison of learning abilities of age-induced memory deficit Swiss Webster mice treated  
10 with the purine derivative AIT-082 and the drug physostigmine;

Fig. 10 is a graphical comparison of learning abilities of age-induced memory deficit C57BL/6 mice treated with the purine derivative AIT-082 and the drug  
15 physostigmine;

Fig. 11 is a graphical comparison of age-induced memory deficit prophylaxis in mice treated with the purine derivative AIT-082 and untreated mice;

Figs. 12A and 12B are graphical comparisons of the  
20 production of nerve growth factor by murine cortical astrocytes in response to the addition of purine derivatives as measured using an ELISA assay; Fig. 12A illustrates measured nerve growth factor concentrations for neurons grown in the presence of different  
25 concentrations of guanosine triphosphate and Fig. 12B illustrates nerve growth factor concentrations for cells grown in the presence of various concentrations of guanosine;

Figs. 13A and 13B are graphical comparisons of the  
30 production of various neurotrophic factor mRNA by murine cortical astrocyte cells grown in the presence and absence of guanosine at different times; Fig. 13A illustrates mRNA levels of nerve growth factor (NGF) and Fig. 13B illustrates mRNA levels of fibroblast growth factor (FGF);

35 Figs. 14A, 14B and 14C are graphical comparisons of neurotogenic responses to different concentrations of

-13-

purine derivative in the presence and absence of nerve growth factor; Fig. 14A illustrates neurotogenic response to various purine derivatives at different concentrations in the presence of nerve growth factor, Fig. 14B illustrates neurotogenic response in the absence of nerve growth factor and Fig. 14C illustrates neurotogenic response to individual purine derivatives and combinations of purine derivatives in the presence and absence of nerve growth factor;

10 Figs. 15A, 15B and 15C are graphical comparisons of nerve growth factor mediated neurotogenic responses in neurons grown in the presence of various concentrations of different purine derivatives; Fig. 15A illustrates neurotogenic response to various concentrations of  
15 inosine; Fig. 15B illustrates the same neurotogenic response to various concentrations of hypoxanthine and Fig. 15C illustrates the neurotogenic response of neuronal cells exposed to different concentrations of xanthine;

Fig. 16 is a graphical representation of nerve growth  
20 factor mediated neuritogenesis measured for neuronal cells grown at various concentrations of the purine derivative AIT-034;

Fig. 17 is a graphical comparison of neurotogenic response of neuronal cells grown at various concentrations  
25 of guanosine triphosphate and adenosine triphosphate with and without nerve growth factor;

Fig. 18 is a graphical comparison of nerve growth factor mediated neurotogenic response to monophosphate, diphosphate, and triphosphate purine derivatives of  
30 guanosine and adenosine;

Fig. 19 is a graphical comparison of cyclic GMP produced in neuronal cells grown in the presence of different concentrations of the purine derivative guanosine;

35 Figs. 20A, 20B and 20C are graphical comparisons of nerve growth factor mediated neurotogenic responses of

-14-

cells grown with and without the purine derivative guanosine in the presence of various concentrations of three different inhibitors; Fig. 20A illustrates the neurotogenic response of cells grown in the presence of methylene blue, a guanylyl cyclase inhibitor, Fig. 20B illustrates the neurotogenic response of cells grown in the presence of various concentrations of LY83583, also an inhibitor of guanylyl cyclase, Fig. 20C illustrates the neurotogenic response of cells grown in the presence of various concentrations of atrial natriuretic factor, a hormone which interacts with guanylyl cyclase;

Fig. 21 is a graphical representation of nerve growth factor-mediated neurotogenic responses for neurons grown in the presence of sodium nitrate, an inorganic nitric oxide donor;

Figs. 22A and 22B are graphical comparisons of nerve growth factor mediated neurotogenic response of neurons grown in the presence of nitric oxide donors and scavengers of nitric oxide and carbon monoxide; Fig. 22A shows the neurotogenic response of cells grown in the presence of various combinations of nitric oxide donors and hemoglobin and Fig. 22B shows the neurotogenic response of cells grown in the presence of various combinations of nitric oxide donors and methemoglobin;

Fig. 23 is a graphical comparison showing the nerve growth factor mediated neurotogenic response of cells grown in various concentrations of hemoglobin with or without the purine derivative guanosine;

Fig. 24 is a graphical comparison showing the nerve growth factor mediated neurotogenic response of cells grown in various concentrations of L-nitro arginine methylester (L-NAME) with and without the purine derivative guanosine;

Fig. 25 is a graphical comparison of the nerve growth factor mediated neurotogenic response for cells grown in the presence of various concentrations of zinc protopor-



-15-

phyrin IX (ZnPP), an inhibitor of CO synthesis, with and without guanosine;

Fig. 26 is a negative control for the graphical comparison shown in Fig. 25 and is a graphical comparison  
5 of nerve growth factor mediated neurotogenic response for cells grown in various concentrations of copper protoporphyrin IX (CuPP), with and without the purine derivative guanosine; and

Fig. 27 is a graphical representation of the nerve  
10 growth factor mediated neurotogenic response for neuron cells grown in the presence of various concentrations of the purine derivative inosine pranobex.

#### DETAILED DESCRIPTION

In a broad aspect, the present invention is directed  
15 to methods and associated compositions and medicaments for use in uniquely treating mammalian cells and neurons to modify cellular or neural activity. More specifically, the present invention is directed to the use of effective purine derivatives to modulate the carbon dioxide  
20 dependent guanylyl cyclase regulatory system within cells or neurons to produce a variety of beneficial results, including the inducement of *in vivo* genetic expression of naturally occurring neurotrophic factors and the resultant direct administration of such naturally occurring  
25 genetically encoded molecules to a mammal, the endogenous production of antioxidant compounds and carbon monoxide, and the resultant ability to reduce mammalian blood pressure. Preventing degenerative cellular destruction and treating disease conditions associated with cellular  
30 damage due to oxidative stress by free radicals also can be achieved with the methods and compositions of the present invention.

In exemplary embodiments illustrative of the teachings of the present invention, particular purine derivatives  
35 were used to induce genetic expression of encoded

-16-

molecules, to stimulate neuritogenesis, to enhance neuronal growth and to modify the membrane potential of neurons to produce increased learning capabilities in mammals. Exemplary studies and treatments were performed  
5 as discussed below using various dosages and routes of administration of selected exemplary purine derivatives representative of compositions that are effective with the methods of the present invention. Of course, those skilled in the art will recognize that the present  
10 invention is not specifically limited to the particular compositions, dosages or routes of administration detailed below.

Depending upon the particular needs of the individual subject involved, the compositions used may be  
15 administered as medicaments in various doses and regimens to provide effective treatment concentrations based upon the teachings of the present invention. What constitutes an effective amount of the selected composition will vary based upon such factors including the activity of the  
20 selected purine derivative, the physiological characteristics of the subject, the extent and nature of the subject's neurodegradation or disorder and the method of administration. Exemplary treatment concentrations which have proven effective in modifying neural activity range  
25 from less than 1  $\mu$ M to concentrations of 500 mM or more. Generally, initial doses will be modified to determine the optimum dosage for treatment of the particular mammalian subject. The compositions may be administered using a number of different routes including orally, topically,  
30 transdermally, intraperitoneal injection or intravenous injection directly into the blood stream. Of course, effective amounts of the purine derivatives may also be administered through injection into the cerebrospinal fluid or infusion directly into the brain, if desired.

35 The methods of the present invention may be effected using purine derivatives administered as medicaments to a

-17-

mammalian subject either alone or in combination as a pharmaceutical formulation. Further, the purine derivatives may be combined with pharmaceutically acceptable excipients and carrier materials such as inert  
5 solid diluents, aqueous solutions or non-toxic organic solvents. If desired, these pharmaceutical formulations may also contain preservatives and stabilizing agents and the like.

The methods and medicaments of the present invention  
10 provide for the controlled long term modification of various types of cellular or neural activities including the *in vivo* production of naturally occurring genetically encoded molecules such as heme oxygenase and neurotrophic growth factors (including neurotrophins, pleiotrophins and  
15 cytokines), the direct administration of such *in vivo* produced molecules, enhancing the effects of these molecules and neurotrophic factors, and the stimulation of cell growth, function, protection, and development. Further, the present invention may be used to promote  
20 neuritogenesis, to form collateral nerve circuits, to enhance the production of cyclic purine nucleotides, to enhance synapse formation and to alter the membrane potential of the neuron. These effects may be extremely beneficial in treating neurodegeneration and in increasing  
25 learning capacity. Similarly, inducing the *in vivo* production of naturally occurring endogenous antioxidant compounds may be extremely useful in treating and preventing disease conditions associated with oxidative damage including cancer, aging, and a variety of  
30 neurological disorders.

For obvious practical and moral reasons, initial work in humans to determine the efficacy of experimental compositions and methods with regard to such afflictions is unfeasible. Accordingly, in the early development of any  
35 drug or therapy it is standard procedure to employ appropriate animal models for reasons of safety and expense.

-18-

The success of implementing laboratory animal models is predicated on the understanding that the cellular or neurophysiology of mammals is similar. Thus, a cellular or neurotropic response in a member of one species, for example, a rodent, frequently corresponds to the same reaction in a member of a different species, such as a human. Only after the appropriate animal models are sufficiently developed will clinical trials in humans be carried out to further demonstrate the safety and efficacy of a therapeutic agent in man.

With regard to neurodegenerative diseases and disorders and to their clinical effects, the mouse model closely resembles the human pathology of these conditions in many respects. Accordingly, it is well understood by those skilled in the art that it is appropriate to extrapolate the mouse or "murine" model to humans and to other mammals. As with humans, mice are susceptible to learning disorders resulting from neuronal degradation, whether due to traumatic injury, oxidative damage, age, disease or harmful chemical agents. Just as significantly, neurotropic factors appear to act in substantially the same manner in a murine model as they do in humans with remarkably similar neuronal reactions. Accordingly, for purposes of explanation only and not for purposes of limitation, the present invention will be primarily demonstrated in the exemplary context of mice as the mammalian subject. Those skilled in the art will appreciate that the present invention may be practiced with other mammalian subjects, including humans, as well.

As will be shown by the data herein, several purine derivatives have been found to work effectively in accordance with the teachings of the present invention. In particular, the data shows that guanosine appears to work well in stimulating the production of neurotrophic factors and enhancing neuritogenesis. Similarly another exemplary purine derivative, 4-[3-(1,6-dihydro-6-oxo-9-

-19-

purin-9-yl)-1-oxopropyl]amino] benzoic acid (AIT-082) has been shown to stimulate the *in vivo* activation or derepression of naturally occurring genes and the resultant production of naturally occurring genetically encoded  
5 molecules such as neurotrophic factors. It also induces the endogenous production of heme oxygenase which in turn induces the endogenous production of carbon monoxide and bile pigment antioxidant compounds. AIT-082 also increases neuritogenesis, enhances the effects of  
10 neurotrophic factors and alters the membrane potential of neurons thereby facilitating long term potentiation of the cells.

AIT-082 is disclosed in U.S. Patent 5,091,432 issued February 25, 1992 to a co-inventor of the present appli-  
15 cation and incorporated herein by reference. Yet another exemplary composition which has been shown to be suitable for use in the present invention is inosine pranobex or isoprinosine. Inosine pranobex, a mixture of inosine and DIP-PacBa at a 1:3 molar ratio was found to enhance neur-  
20 itogenesis and the effects of neurotrophic factors *in vitro*. The different embodiments of the present invention presented above demonstrate the applicability of using various purine derivatives to modify cellular and neural activity through modulating the carbon monoxide dependent  
25 guanylyl cyclase system.

Exemplary preferred embodiments of the present invention involve the treatment of cells or neurons with AIT-082 or 4-[3-(1,6-dihydro-6-oxo-9-purin-9-yl)-1-oxopro-  
pyl]amino] benzoic acid. As discussed previously, AIT-082  
30 is a unique derivative of the purine hypoxanthine containing a para-aminobenzoic acid moiety. It is rapidly absorbed after oral administration and, after crossing the blood brain barrier, enters the brain unchanged. It may be detected at levels as high as 3.3 ng/mg brain tissue  
35 minutes after oral administration. AIT-082 induces the *in vivo* genetic expression of naturally occurring genetically

-20-

encoded molecules including heme oxygenase and neurotrophic factors. As a result, the present invention is able to induce the direct self-administration of these compounds to the treated cells and to stimulate the associated metabolic pathways and resultant physiological effects. It also stimulates neurite outgrowth from neuronal cells when added alone to the cultures as well as enhancing the neurotogenic effects of neurotrophic factors such as nerve growth factor (NGF). More importantly, AIT-082 enhances working memory in old, memory deficient mice after either intraperitoneal and oral administration.

The neurotogenic activity of AIT-082 is inhibited by hemoglobin, by Methylene Blue, and by ZnPP, all scavengers of CO, but not by CuPP or by other inhibitors of nitric oxide synthase. Screening tests for *in vitro* activity at known neurotransmitter and neuromodulator receptors were negative. ZnPP is also known as an inhibitor of heme oxygenase I which identifies the site of action of this exemplary guanylyl cyclase modulating purine derivative. Heme oxygenase is a known heat shock (stress) protein. Heat shock proteins have been demonstrated to regulate the conformation, intracellular transport, and degradation of intracellular proteins. They are also involved in maintaining cellular viability during stress. Some in the art believe that decreased levels or functioning of heat shock (stress) proteins may be one of the factors leading to increased deposition of abnormally folded proteins and cell death in the brains of Alzheimer's patients. Thus, the ability of AIT-082 to induce the *in vivo* production of heme oxygenase evidences the impact of the present invention on a variety of protective cellular pathways, indicating the therapeutic applicability of the present invention to the treatment of heart attack and stroke in addition to treating other disease conditions.

A further understanding of the present invention will be provided to those skilled in the art from the following

-21-

non-limiting examples which illustrate exemplary protocols for the identification, characterization and use of purine derivatives in accordance with the teachings of the present invention.

5

**EXAMPLE 1****PLASMA LEVELS OF AIT-082 IN MICE**

Adult C57BL/6 mice were administered 30 mg/kg of AIT-082 in saline i.p. The animals were sacrificed by decapitation at 30, 45, 60 and 90 minutes after administration of AIT-082. Blood was collected in heparinized tubes, mixed and centrifuged at 2000 rpm for 15 minutes. The plasma supernatant was removed and stored at -70°C until analysis. A high pressure liquid chromatography system was developed for the analytical measurement of AIT-082 in plasma and brain tissue. The assay developed was selective for AIT-082 in the presence of a number of closely related purine molecules. The sensitivity of the method was 0.1 microgram of AIT-082 per ml of plasma and 0.1 microgram of AIT-082 per milligram of brain tissue (wet weight).

The results of these determinations are shown in Table A and graphically represented in Fig. 1 where plasma levels of AIT-082 are provided at 30, 45 and 60 minutes after administration of 30 mg/kg i.p. to C57BL/6 mice. From the data, it was estimated that the blood level of AIT-082 reached its peak at approximately 45 minutes and a plasma elimination half-time of approximately 12 minutes with the  $k_d = 3.45 \text{ hr}^{-1}$ .

-22-

**Table A**  
**Plasma Levels of AIT-082**

	Time (min)	Level ( $\mu\text{g/ml} \pm \text{S.E.}$ )
5	15	42 $\pm$ 6
	30	108 $\pm$ 13
	45	437 $\pm$ 131
	60	86 $\pm$ 24
	90	20 $\pm$ 12

10

**EXAMPLE 2****AIT-082 CROSSES THE BLOOD BRAIN BARRIER**

Brain tissue was analyzed from two animals receiving 30 mg/kg i.p. of AIT-082 and sacrificed 30 minutes after drug administration. The brains were rapidly removed and  
15 chilled on ice. Brain tissue was dissected into cortex and remainder of the brain. Brain tissue (approx. 250-300 mg wet weight) was homogenized with 5.0 ml of saline using a Brinkman Polytron tissue grinder and stored at  $-70^{\circ}\text{C}$  until analysis. Brain homogenates were deproteinized by  
20 ultrafiltration through Gelman Acrodisc filters; first through a 1.2 micron filter and then through a 0.2 micron filter. A 30  $\mu\text{l}$  sample was injected into the HPLC for analysis as above. A standard curve was prepared by the addition of known quantities of AIT-082 to brain  
25 homogenates from untreated animals. Analysis of the brain tissue indicated that AIT-082 was detected in both the cortex sample and the remaining brain samples from both animals. The results are shown directly below in Table B.



-23-

**Tabl B**  
**Brain Tissue Levels of AIT-082**

Sample #	Brain Region	Brain wt (mg)	Level of AIT-082 (ng/mg brain tissue)
5 S3	Cortex	181	2.8
S3	Remainder	153	3.3
S4	Cortex	146	3.4
S4	Remainder	217	2.3

This demonstration of the presence of AIT-082 in the brain tissue after 30 minutes is critical in that it indicates that AIT-082 crosses the blood-brain barrier without degradation.

### EXAMPLE 3

#### AIT-082 INTERACTS WITH THE CHOLINERGIC SYSTEM

Because of the finding that there is a severe loss of cholinergic neurons in the hippocampus in Alzheimer's disease patients, there has been considerable interest in the effect on memory of compounds which alter the activity of this system. Support for the cholinergic hypothesis of memory comes from studies using lesions or a stroke model. Lesions of the CA1 region of the hippocampus appear to specifically disrupt working memory. In the stroke model, occlusion of the vertebral and carotid arteries (30 minutes) produces specific cell loss in the CA1 region of the hippocampus and a loss of working memory. In these models in aged rats, physostigmine, a cholinesterase inhibitor, has been shown to improve memory. THA, another drug which increases cholinergic function, was shown to improve memory in aged monkeys. The observation that AIT-082 improves memory in the same general manner as physostigmine and THA raises the question of whether AIT-082 might have some effect on the cholinergic system.

To elucidate the mechanisms by which AIT-082 improves memory, attempts were made to block its actions by co-

-24-

administration of the short-acting cholinergic antagonist atropine to mice and subjecting them to simple learning tests. Atropine reportedly has the ability to block the effects of physostigmine and THA. Mice were injected with  
5 AIT-082 (30 mg/kg) 2 hr prior to testing on days 1 through 4. Atropine (0.5 mg/kg) (28), was injected 1/2 hour prior to testing or 1.5 hours after AIT-082 on day 3 only. All injections were i.p. After a reference run to determine where the reward was placed in a T-maze, the mice were  
10 retested to determine if they could remember the location of the reward. The percentage of correct responses is graphically represented in Fig. 2.

Fig. 2 demonstrates that atropine blocked the memory enhancing activity of AIT-082 on day 3 and that the effect  
15 was transient since the enhancing effects of AIT-082 reappeared on day 4 when no atropine was administered. This observation suggests that a cholinergic mechanism may be involved in the action of AIT-082.

#### EXAMPLE 4

##### 20 EFFECT OF AIT-082 ON ACETYLCHOLINE RECEPTORS

The interaction of AIT-082 with acetylcholine receptors was determined by interference with the binding of QNB (3-quinuclidinyl benzilate) in mouse tissue using the method of Fields [J.Biol. Chem. 253(9): 3251-3258, 1978].  
25 There was no effect of AIT-082 in this assay.

In the study, mice were treated with AIT-082 at 30 mg/kg 2 hours prior to sacrifice, decapitated and the tissue processed to obtain membranes containing the acetylcholine receptors. When these tissues were assayed  
30 in vitro, there was no effect of AIT-082 on affinity (Kd) for QNB when AIT-082 was administered under the same conditions as utilized in testing for effects on memory. There was a change in the number of receptors (B max) in cortex and striatum, with the cortex showing a decrease  
35 and the striatum an increase in acetylcholine binding

-25-

sites. These data are consistent with the hypothesis that there is an increase input to the cortex as a result of AIT-082 being administered to the animals. Typically, an increased input will result in down regulation of the  
5 receptors.

#### EXAMPLE 5

##### EFFECT OF AIT-082 ON RECEPTOR LIGAND BINDING IN VITRO

AIT-082 was evaluated for its ability to inhibit ligand binding to 38 isolated receptors. The receptors  
10 screened and their ligands were:

Adenosine

Amino Acids:

Excitatory Amino Acids (glycine, kainate, MK-801, NMDA, PCP, quisqualate and sigma);

15 Inhibitory Amino Acids (benzodiazepine, GABA-A, GABA-B, and glycine)

Biogenic Amines (dopamine-1, dopamine-2, serotonin-1, serotonin-2)

20 Calcium Channel Proteins (nifedipine, omegaconotoxin, chloride, potassium)

Peptide Factors ( ANF, EGF, NGF)

25 Peptides: (angiotensin, arg-vasopressin-V1 and V2, bombesin, CCK central and peripheral, neurotensin, NPY, somatostatin, substance K, substance P, VIP)

Second Messenger Systems:

Adenyl Cyclase

Protein Kinase (phorbol ester and inositol triphosphate)

30 The testing was conducted under contract at Nova Labs (Baltimore, MD). AIT-082 had no activity in any of the in vitro assays conducted.

Accordingly, while AIT-082 acts through the cholinergic nervous system (atropine blocks its activity),  
35 AIT-082 appears to act through a mechanism that does not

-26-

involve direct interaction with acetylcholin receptors. It is of importance to note that *in vitro*, AIT-082 does not bind to the adenosine receptor.

AIT-082 was evaluated in a series of psychopharmacological tests that were established in order to more fully evaluate the scope of its central nervous system activity. Among the tests utilized were:

- (a) motor coordination, by the accelerating Rota-Rod treadmill,
  - 10 (b) exploratory and home cage locomotor activity, by the Stoelting activity monitor,
  - (c) anxiolytic activity, by the elevated Plus maze, and
  - (d) nociception.
- 15 AIT-082 was compared with standard reference drugs.

#### EXAMPLE 6

##### AIT-082 INCREASES MOTOR COORDINATION IN MICE

Motor coordination was measured using an accelerating Rota-Rod treadmill for mice (Ugo Basile Co.). At various  
20 times after treatment with saline or drug, mice were placed on the Rota-Rod, which accelerates to maximum speed over a 5 minute period. The time in seconds at which the subject falls off was recorded in Table C directly below. Each animal was tested 3 times and the mean time was  
25 recorded.

-27-

**Table C**  
*Effect of AIT-082 on Roto-rod performance*

	AIT dose (mg/kg)	Time (sec)
5	Control	123±64
	0.005	162±93
	0.05	207*±73
	0.5	184*±76
	30.0	187*±68
10	60.0	229*±80

\*p<0.05, t-test vs controls

Subjects receiving AIT-082 showed improved motor coordination by remaining on the roto-rod for longer periods of time when compared to control (saline) or low doses (0.005 mg/kg).

#### EXAMPLE 7

##### AIT-082 DOES NOT INHIBIT EXPLORATORY ACTIVITY

To measure exploratory behavior, subjects received saline or AIT-082 administration, were placed in a novel large cage (25x48x16 cm, WxLxH), and movement was measured at one-minute intervals for 30 minutes. The large cage (San Diego Instruments, San Diego, California) was equipped with vertical detectors and rearing movements were also recorded. No effects were noted with respect to exploratory activity indicating that the subjects were not incapacitated.

#### EXAMPLE 8

##### AIT-082 DOES NOT INHIBIT LOCOMOTOR ACTIVITY

To measure home cage locomotor activity, the home cage was placed on a platform of an activity monitor (Stoelting Instruments). Home cage locomotor activity movements were recorded at one minute intervals for 15 minutes. Subjects received saline or AIT-082 and were returned to their home

-28-

cages. Ten minutes after injection, the home cage was replaced on the platform of the activity monitor. Home cage locomotor activity movements were recorded at one minute intervals for 30 minutes. During the first five minutes, grooming activity was also monitored and recorded. The results are shown in Table D directly below.

**Table D**  
*Effect of AIT-082 on locomotor activity*

10	AIT dose (mg/kg)	Movements (mean $\pm$ S.D.)		
		Pre-drug	Post-drug	Difference
	Control	1633 $\pm$ 434	1385 $\pm$ 492	248 $\pm$ 492
	0.005	1884 $\pm$ 230	1375 $\pm$ 563	509 $\pm$ 429
	0.05	1718 $\pm$ 606	1508 $\pm$ 456	209 $\pm$ 340
	0.5	1610 $\pm$ 349	1320 $\pm$ 689	290 $\pm$ 435
15	30.0	1440 $\pm$ 264	1098 $\pm$ 189	342 $\pm$ 267
	60.0	1690 $\pm$ 223	634* $\pm$ 223	1056* $\pm$ 154

\*p<0.05, t-test vs controls

As shown by the data in Table D, at the high dose (60 mg/kg), subjects may have become more habituated to their environment and exhibited less movement after treatment with AIT-082. Otherwise, no effects were noted.

#### EXAMPLE 9

##### AIT-082 DOES NOT SUBSTANTIALLY INCREASE ANXIETY

A Plus maze was constructed of black plexiglass consisting of two opposite-facing open arms (30x5 cm, LxW) and two opposite facing closed arms (30x5x15 cm, LxWxH). The walls of the closed arms were clear plexiglass and the four arms were connected by a central area 5x5 cm. The entire Plus maze was mounted on a base 38 cm above the floor. Testing consisted of placing the subject at one end of one of the open arms. The time the subject took to leave the start position (the first 10 cm of the open

-29-

arm) was recorded. The time it took for the subject to enter halfway into one of the closed arms was also recorded. When the subject arrived at the half-way point in the closed arm, the three-minute test session began.

5 During the three-minute test session, the number of times the subject entered the open arms was recorded. An entry was defined as placing at least two paws onto the platform of the open arm. There was a slight anxiogenic effect of AIT-082 at 30 mg/kg, but this was not observed at a higher

10 dose (60 mg/kg) or at the lower doses (0.005 to 0.5 mg/kg).

#### EXAMPLE 10

##### AIT-082 DOES NOT EFFECT NOCIOCEPTION

Mice were placed on an electric hot plate (Omnitech)

15 at 55°C and the latency time until the subject licked his hind paw was measured. If there was no response by 45 seconds, the trial was terminated. By this test there was no effect of AIT-082 on nociception.

#### EXAMPLE 11

##### AIT-082 IS NOT TOXIC

Preliminary acute toxicity tests in rats and mice of AIT-082 have demonstrated that the LD<sub>50</sub> is in excess of 3000 mg/kg when administered by the oral or intraperitoneal route. AIT-082 has been evaluated under Panlabs's

25 General Pharmacology Screening Program (Panlabs, 11804 North Creek Parkway South, Bothwell, Washington 98011) and the results indicated an absence of any toxicity when measured in their standard profile of 79 different test systems.

30 By the nature of the chemical structure of AIT-082, it is not anticipated that the compound will be metabolized into any toxic metabolites.

-30-

In conclusion, there were few deleterious effects of AIT-082 on a variety of psychopharmacological tests except for a slight anxiogenic effect at one dose. There was an increase in motor coordination (roto-rod test) over a range of doses (0.05 to 60 mg/kg) and possibly a learning or habituation effect at one dosage (60 mg/kg) in the locomotor test.

Following psychopharmacological characterization of this exemplary compound, further studies were conducted to demonstrate the neurogenic effects of the present invention.

#### EXAMPLE 12

##### AIT-082 PROMOTES NEURITOGENESIS IN PC12 CELLS

Much of the work performed in the characterization of the compounds of the present invention involved the use of PC12 cells. These cells are derived from a rat pheochromocytoma and when grown in the presence of NGF, extend neurites, cease cell division and assume many characteristics of sympathetic neurons. When cultured in the absence of nerve growth factor (NGF), few PC12 cells have neurites greater than one cell diameter. Addition of saturating concentrations of NGF for 48 hours stimulates neurite outgrowth in about 20-35% of the cells. Because they constitute a homogeneous population of neuronal-like cells, without contaminating astroglia type cells, it is possible to study the direct effects of the purine based compounds on neurite outgrowth in these cells.

To demonstrate neuronal modification by the exemplary compounds of the present invention, a dose response curve of AIT-082 was generated measuring the stimulation of neuritogenesis in PC12 cells. Cells cultured in RPMI 1640 with 1.5% horse serum and 1.5% fetal bovine serum were replated onto poly-ornithine coated 24-well culture plates ( $2.5 \times 10^4$  cells per well). AIT-082 and NGF were added to the various cultures immediately upon plating. After 48



-31-

hours, medium was removed and the cells immediately fixed in 10% formalin and PBS for 10 minutes. Cells and neurites were counted within 2 days of fixation.

A neurite was defined as a process extending from the cell at least 1 cell body diameter in length and displaying a growth cone at its tip. For each treatment, 2 representative microscope fields were counted from each of 6 sister cultures receiving identical treatments. The total number of cells counted per well (approximately 100 cells) and the total number of cells containing neurites in each well were used to determine fraction of neurite-bearing cells. The mean values ( $\pm$  SEM) were then determined for each of the treatments. To facilitate comparison neurite outgrowth was expressed relative to the proportion of cells bearing neurites in the presence of NGF alone (NGF=100%). The effects of compounds with and without NGF were compared by analysis of variance (ANOVA) followed by Tukey's test for significance.

The results are shown in Fig. 3 where the curve represents different levels of AIT-082 plus saturating concentrations (40 ng/ml) 2.5 S NGF. The center horizontal line represents control values for cells cultured in the presence of 40 ng/ml NGF alone. Upper and lower horizontal lines are indicative of confidence limits of NGF alone as determined using standard statistical methods.

As shown in Fig. 3, AIT-082 stimulates neuritogenesis and enhances NGF-stimulated neuritogenesis in PC12 cells at low concentrations (1  $\mu$ M). Analysis of the data shows that AIT-082 was as effective as NGF in promoting neuritogenesis in PC12 cells and enhanced the optimal effects of NGF by 30%. For the purposes of comparison, and as will be discussed in more detail below, inosine and hypoxanthine are weakly effective in stimulating neuritogenesis and in enhancing NGF-stimulated neuritogenesis in PC12 cells but are effective at lower concentra-

-32-

tions of 30-300 nM. Guanosine produces a significant effect similar to AIT-082 but at a higher concentration of 30-300  $\mu$ M.

### EXAMPLE 13

#### 5 EFFECT OF INHIBITORS ON AIT-082 NEURITOGENESIS

Age-related memory loss has been associated with loss of NGF-dependent basal forebrain neurons. It can be ameliorated by i.c.v. infusion of NGF. The effect of AIT-082 on neuritogenesis alone and with NGF were studied using  
10 the protocol of Example 12. In order to study the mechanism by which AIT-082 exerts its effects, a series of experiments were conducted in which inhibitors were utilized to block or modify specific biochemical processes. All of the cultures contained NGF at optimal dose  
15 (40 ng/ml) so the series without AIT-082 added represented the effect of the inhibitors on NGF activity. Where indicated, AIT-082 was added at 10 $\mu$ M, its apparent, presently understood, optimal dose. Three selective inhibitors were used.

20 The results of these studies are shown below in Table E below, and Figs. 4A, 4B, and 4C graphically present the proportion of cells bearing neurites after 48 hours culture under the conditions indicated. The base line value was cells grown without NGF or AIT-082.

-33-

**Table E**  
*Effect of AIT-082 and selective inhibitors on  
 neuritogenesis alone and with NGF*

Inhibitor	Concentration	AIT-082 alone <sup>1</sup>	NGF alone	AIT-082+NGF
5 None		0.2 ± 0.02	0.2 ± 0.02	0.26 ± 0.01
Methemoglobin	0		0.2 ± 0.02	0.26 ± 0.01
	1 μM		0.2 ± 0.02	0.17 ± 0.02
Methylene Blue	0		0.2 ± 0.02	0.26 ± 0.01
	5 μM		0.24 ± 0.03	0.10 ± 0.01
Zn Protoporphyrin IX	0		0.20 ± 0.02	0.26 ± 0.01
	1 μM		0.22 ± 0.03	0.13 ± 0.01

10 <sup>1</sup>Proportion of cells bearing neurites

Methemoglobin (MHb) captures and removes nitric oxide (NO) and carbon monoxide (CO) from the culture media. MHb had no effect on NGF activity but inhibited the action of AIT-082, implying that either NO or CO is involved in the  
 15 action of AIT-82.

Methylene blue (MB) inhibits soluble guanylyl cyclase, the enzyme which produces cyclic GMP (cGMP) a intracellular substance which, as previously discussed, is involved in the second messenger system of nerve impulse transmission. MB had no effect on NGF activity but inhibited the  
 20 action of AIT-082, implying that guanylyl cyclase is involved in the mechanism of action of AIT-082.

Zinc protoporphyrin IX (ZnPP) is an inhibitor of heme oxygenase II (HO) which in turn produces CO. ZnPP had no  
 25 effect on NGF activity but inhibited the action of AIT-082. This identifies a potential site of action of AIT-082 as involving the production of HO. It also identifies the resultant production of CO as part of the mechanism of action of AIT-082. These results are  
 30 indicative of a number of important aspects and features of the present invention.

-34-

As previously discussed, HO is a heat shock (stress) protein. These proteins are believed to be part of protective mechanisms necessary to maintain cellular viability during stress (see, e.g. Georgopoulos, C. and W.J. Welch, *Role of the major heat shock proteins as molecular chaperones*. *Annu. Rev. Cell Biol.*, 1993. 9: p. 601-634). In addition to regulating the confirmation, intracellular transport, and degradation of intracellular proteins, HO is also strongly implicated in the production of potent antioxidant compounds produced by the degradation of heme through the enzymatic activity of HO. In mammalian tissue the sole source of the protective antioxidant bile pigments biliverdin and bilirubin is heme degraded by HO. There is substantial evidence that these bile pigments play an important physiological role in cellular antioxidant defense mechanisms (Stocker, P., et al., *Bilirubin is an antioxidant of possible physiological importance*. *Science*, 1987. 235: p. 1043-1047). Moreover, HO is found in the brain, and its level is known to greatly increase after heat shock (Ewing, J.F., S.N. Haber, and M.D. Maines, *Normal and heat-induced patterns of expression of heme oxygenase-1 (HSP32) in rat brain: hyperthermia causes rapid induction of mRNA and protein*. *J. Neurochem.*, 1992. 58: p. 1140-1149).

Consistent with this understanding, a number of neurotrophins exert their neuro-protective effects by stimulating endogenous defenses against oxidative stress and damage by free radicals (Williams, L.R., *Oxidative stress, age-related neurodegeneration, and the potential for neurotrophic therapy*, in *Cerebrovasc. Brain Metab. Rev.* 1995, Raven Press, Ltd.: New York, NY. p. 55-73. Mattson, M.P., B. Cheng, and V.L. Smith-Swintosky, *Mechanisms of neurotrophic factor protection against calcium and free radical-mediated excitotoxic injury: implications for treating neurodegenerative disorders*. *Exp. Neurol.*, 1993. 124: p. 89-95). Because cytotoxic

-35-

free radicals are suspected in the etiology of a variety of neurodegenerative diseases, it is reasonable to conclude that the present invention is able to stimulate the production or activity of HO to produce protective  
5 antioxidant compounds which function to prevent degenerative cell destruction by oxidative free radicals through the neutralization or sequestration of these toxic oxidative compounds.

Currently, it is believed by many skilled in the art  
10 that ALS, Parkinson's disease, and Alzheimer's disease may result from an inability to protect against accumulated cellular damage by free radicals. Some practitioners skilled in the art have experimented with the treatment of ALS through the administration of neurotrophins  
15 (DiStefano, P.S., *Neurotrophic factors in the treatment of motor neuron disease and trauma*. Exp. Neurol., 1993. 124: p. 56-59. Thoenen, H., R.A. Hughes, and M. Sendtner, *Trophic support of motoneurons: Physiological, pathophysiological, and therapeutic implications*. Exp.  
20 Neurol., 1993. 124: p. 47-55). Because the present invention is able to endogenously produce these protective compounds it provides an effective treatment for these and other degenerative cellular conditions. This protective ability is particularly important for the treatment of  
25 neurons because, unlike most cells which possess a variety of protective mechanisms, such as high levels of glutathione, neurons are deficient in this antioxidant source.

The ability of the present invention to stimulate the  
30 activity or production of HO has at least one additional direct physiological benefit in mammals. When heme is degraded by HO into the bile pigments bilirubin and biliverdin, CO is also produced. Thus, the ability of the present invention to stimulate HO production and activity  
35 also provides the present invention with the ability to stimulate the *in vivo* cellular production of CO. CO is

-36-

known as an activator of soluble guanylate cyclase and relaxes vascular smooth muscle via a cGMP-dependent mechanism (Graser, T., Y.P. Verdernikov, and D.S. Li, *Study of the mechanism of carbon monoxide induced endothelium-independent relaxation in porcine coronary artery and vein*. Biomed. Biochim. Acta, 1990. 49: p. 293-296. Morita, T., et al., *Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP*. Proc. Natl. Acad. Sci. USA, 1995. 92: p. 1475-1479). Recently, it was demonstrated by others in the art that withdrawal of the blood pressure lowering effects of CO through inhibition of HO resulted in an increase in mammalian blood pressure (Johnson, R.A., et al., *A heme oxygenase product, presumably carbon monoxide, mediates a vasodepressor function in rats*. Hypertension, 1995. 25: p. 166-169). Accordingly, it is reasonable to conclude that the ability of the present invention to stimulate the production of CO in vivo will reduce mammalian blood pressure in addition to providing increased antioxidant protection. The ability of the present invention to directly induce the in vivo production of naturally occurring cellular compounds producing these dramatic physiological effects is unprecedented in the art.

#### EXAMPLE 14

##### 25 EFFECT OF NITRIC OXIDE INHIBITORS ON AIT-082

Nitric oxide is produced by the action of the enzyme nitric oxide synthetase (NOS). Two chemicals that have been shown to selectively inhibit NOS are N-nitro-L-arginine methyl ester (L-NAME) and N-nitro-L-arginine (NOLA). Different levels of these chemicals were administered simultaneously with AIT-082 and neuriteogenesis in PC12 was measured using the protocol of Example 12. The results for L-NAME are presented in Table F while the results for NOLA are presented in Table G. Both tables

are shown directly below with graphical representations of the data presented in Figs. 5A and 5B.

**Table F**  
*The effect of L-NAME on neuritogenesis*

		Concentration of L-NAME ( $\mu$ M)			
5	AIT-082	None	0.1	1.0	10.0
	0	0.246 $\pm$ 0.017	0.259 $\pm$ 0.027	0.257 $\pm$ 0.013	0.251 $\pm$ 0.013
	10 $\mu$ M	0.254 $\pm$ 0.008	0.220 $\pm$ 0.010	0.302 $\pm$ 0.027	0.254 $\pm$ 0.018
	100 $\mu$ M	0.309 $\pm$ 0.027	0.257 $\pm$ 0.016	0.232 $\pm$ 0.019	0.289 $\pm$ 0.006

**Table G**  
*The effect of NOLA on neuritogenesis*

		Concentration of NOLA ( $\mu$ M)			
10	AIT-082	None	0.1	1.0	10.0
	0	0.246 $\pm$ 0.017	0.259 $\pm$ 0.009	0.311 $\pm$ 0.016	0.305 $\pm$ 0.017
	10 $\mu$ M	0.254 $\pm$ 0.008	0.277 $\pm$ 0.016	0.312 $\pm$ 0.029	0.298 $\pm$ 0.019
	100 $\mu$ M	0.309 $\pm$ 0.027	0.279 $\pm$ 0.027	0.295 $\pm$ 0.028	0.310 $\pm$ 0.023

15 As shown by the data in Tables F and G, neither of these inhibitors of NOS were active in blocking the effect of AIT-082 on neuritogenesis. These results indicate that NO was not involved in the mechanism of action of AIT-082.

#### EXAMPLE 15

##### 20 EFFECT OF AIT-082 ON cGMP LEVELS IN PC-12 CELLS

To demonstrate CO-dependent guanylyl cyclase modification, cyclic guanosine monophosphate (cGMP) levels in PC12 cells were measured following addition of AIT-82. Initially, PC-12 cells were primed with 40 ng/ml NGF for  
25 3 days in low serum medium (1.5% horse serum + 5% fetal

-38-

calf serum). Cells were seeded onto assay plates in low serum medium containing 40 ng/ml NGF and incubated for 1 hour. The medium was changed to low arginine medium (80  $\mu$ M) with no serum and NGF and papaverine (100  $\mu$ M) where  
5 indicated. Test compounds were added for the indicated time and the reaction was stopped by adding 5% TCA containing 10,000 dpm of  $^3$ H-cGMP. cGMP was assayed by the radioimmunoassay method of Maurice [Mol. Pharmacol. 37: 671-681, 1990]. TCA was purified by adding powdered  
10 charcoal (5g) and filtering the mixture through Whatman #1 paper. This removed contaminants in the TCA that otherwise interfere with the radioimmunoassay (RIA) of cGMP.

It was necessary to purify the cGMP from cAMP and other contaminants before radioimmunoassay since these  
15 other materials can interfere with the assay. Briefly, the TCA solution was applied to Dowex columns (50W-8X, 200-400 mesh) and eluted. A neutral alumina column was then placed under each Dowex column. The cGMP was eluted from the Dowex columns into neutral alumina columns by  
20 adding 4mL of 0.05M HCl to each Dowex column. The neutral alumina columns were then sequentially rinsed with 2ml of HCl, 4mL water and finally with 0.2M sodium acetate (pH 6.2). The cGMP collected for the RIA, eluted in 1mL of sodium acetate with a recovery between 50-65%. The cGMP  
25 was assayed using a Dupont RIA kit. The results are graphically presented in Fig. 6.

As shown in Fig. 6, the addition of AIT-082 increased the production of cGMP in PC12 cells indicating that AIT-082 acts by modifying the activity of the carbon  
30 monoxide-dependent enzyme guanylyl cyclase.

#### EXAMPLE 16

##### EFFECT OF AIT-082 ON GENETIC EXPRESSION OF NEUROTROPHIN mRNA

To demonstrate that AIT-082 induced the *in vivo*  
35 genetic expression and resultant cellular production of



-39-

neurotrophins, naturally occurring, genetically encoded molecules, as well as enhancing their activity, the following experiment was performed. Induction of neurotrophin mRNA was determined by northern blot analysis of astrocytes cultured with AIT-082, NGF, or both. The cells were harvested and RNA extracted at 24 hours after treatment.

More particularly, astrocytes from the cerebral cortex of NIH Swiss mice (Harlan) were isolated. Briefly, newborn pups (0-24 hours) were decapitated. Their brains were removed under aseptic conditions and were placed in modified Dulbecco's medium (DMEM) containing 20% heat-inactivated horse serum (Hyclone) - ("complete medium"). The neopallium was then dissected from each cerebral hemisphere and minced into 1mm cubes.

The astrocytes were then isolated by mechanical dissociation. The cubes were vortexed at maximum speed for one minute. The cell suspension was then passed first through 75mm Nitex then through 10mm Nitex. The resulting cell suspension was diluted in complete medium to a final concentration of one brain per 10ml of complete medium. Ten milliliters of the diluted cell suspension, was added to each 100mm Falcon tissue culture plate (Fisher). After 3 days the medium was replaced with 10ml fresh complete medium and subsequently was replaced twice weekly with DMEM containing 10% heat inactivated horse serum - ("growth medium"). After two weeks in culture the astrocytes formed a confluent monolayer.

For RNA extraction, astrocytes were trypsinized. The astrocytes were then replated onto 100 mm PORN coated plates at a cell density of  $10^6$  cells per plate (10 ml growth medium). After 2 hrs PBS, Guo, or GTP at 10mM were added to the appropriate plates. Total RNA was harvested from  $1.5 \times 10^7$  cells for each treatment, 4 and 24 hrs after treatment using TRIzol reagent and supplier protocol (GIBCO BRL/Life Technologies, Inc.). For slot blots,

-40-

total RNA was bound to Hybond-N filters (Amersham/United States Biochemicals) as described in Transfer and Immobilization of Nucleic Acids and Proteins to S & S Solid Supports (S and S protocols: Schleicher & Schuell, New Hampshire, USA). Northern blots were also performed using 10-20 mg total RNA from each sample. These were electrophoresed in 1% agarose gels containing formaldehyde and blotted onto Hybond-N filters according to S and S protocols.

10 The blots were probed with  $P^{32}$ -labelled cDNA (NGF, NT-3 and BDNF probes) or oligonucleotide probe (FGF-2) by hybridization in Piperazine-N,N'-bis-[2-ethanesulfonic acid] (PIPES) buffer (50 mM PIPES, pH 6.8; 50 mM  $NaH_2PO_4$ ; 0.1 M NaCl; 5% SDS and 1 mM EDTA) overnight at 50°C. The blots were then washed twice with (2X SSC, 0.1% SDS) wash buffer at room temperature for 20 minutes each, and then with (0.1X SSC, 0.1% SDS) wash buffer twice at 52°C for 20 minutes each. 1X SSC is 0.15M NaCl and 15 mM sodium citrate, pH 7.0. Damp membranes were wrapped in Saran wrap and autoradiography was performed using Hyperfilm-MP (Amersham/USB) and a cassette with intensifying screens. Various concentrations (0.25 to 4 mg of total RNA), as determined by spectrophotometry, of each sample were blotted and probed so that quantification could be done after insuring a linear film response. Quantification was performed using MCID Image Analysis (St. Catherine's, Ontario, Canada).

To provide probes, a cDNA clone of the mouse NGF gene in the plasmid pGEM.NGF(+), and cDNA clones of human NT-3 and BDNF in Bluescript were isolated. After isolation, the cDNA probes were labeled with  $^{32}P$ -dCTP (ICN Biomedicals Canada, Ltd.) using a Random Primed DNA Labeling Kit (Boehringer Mannheim Biochemica) as described in the kit.

A 40-mer antisense oligonucleotide was synthesized (MOBIX, McMaster University) as a probe for FGF-2. This was complementary to the 5' end of mouse FGF-2 coding

-41-

sequence on the mRNA. The oligo was 5' end-labeled using polynucleotide kinase, One-Phor-All buffer, and the protocol supplied by Pharmacia Biotech Inc., and ATP $\gamma$ P<sup>32</sup> (ICN Biomedicals Canada, Ltd.).

- 5 The results of the study for the production of four different neurotrophic factors are shown below in Table H.

**Table H**  
*Northern Blot analysis of neurotrophin  
mRNAs from Astrocytes*

10	Neurotrophin mRNA	Control	NGF 40 ng/ml	AIT-082 100 mM	AIT-082 (100mM) + NGF (40 ng/ml)
	NGF	-	-	++	+
	FGF-2	+	-	++	+
	BDNF	+	+	+	+
15	NT-3	-	-	++	+

The conditions which produced a detectable amount of each of the neurotrophin mRNAs are indicated by a "+", with a "++" indicating that at least twice the detectable amount was present. Those blots which were negative are indicated by a "-".

- 20 The results indicate that AIT-082 induced the expression of mRNAs for several neurotrophic factors, including NGF. More importantly, these data clearly establish that AIT-082 selectively and controllably induced the *in vivo* genetic expression of at least one naturally occurring
- 25 genetically encoded molecule in a mammal treated in accordance with the teachings of the present invention. Administering this exemplary purine derivative selectively induced the expression of mRNA encoding three of the four identified neurotrophic factors, NGF, FGF-2, and NT-3, but
- 30 did not induce activation or derepression of the gene encoding for BDNF mRNA. This selective control coupled with the ease of administration provided by the compounds and

-42-

methods of the present invention effectively overcomes the limitations of the prior art. Rather than administering these molecular compounds directly to cells through complex and potentially dangerous techniques, the present invention is able to treat a mammalian patient utilizing traditional, noninvasive drug delivery routes that induce the treated cells to express the genetic material encoding the desired compounds resulting in their direct *in vivo* delivery and administration. Though potentially useful in conjunction with modified genes or other molecular biology techniques, with the present invention, genetic modification is unnecessary.

It has been shown previously that, within the hippocampus from Alzheimer's patients, there is an altered program of gene expression leading to aberrant levels of mRNA for neurotrophic factors. A number of animal and clinical studies have demonstrated that administration of single neurotrophins are inadequate to treat neurodegenerative disease. Accordingly, the ability of the compounds of the present invention to stimulate the production of multiple neurotrophin mRNAs within cells substantially increases their potential as treatments for a variety of neurodegenerative diseases by providing a method for the effective direct administration of these naturally occurring genetically encoded molecules to a patient through the induction of their *in vivo* genetic expression.

The preceding examples show that AIT-082 stimulates neuriteogenesis *in vitro* in PC12 cells alone and enhances the effect of nerve growth factor (NGF). Further, the neurotogenic effect of AIT-082 was reduced by methemoglobin (which captures and removes nitric oxide and carbon monoxide), methylene blue (which inhibits guanylyl cyclase), and by zinc protoporphyrin IX (an inhibitor of heme oxygenase, which produces carbon monoxide). The neurotogenic effect of AIT-082 was unaffected by L-NAME or

-43-

NOLA, inhibitors of NO production. In addition, AIT-082 stimulated the production of a number of different neurotrophic factors as evidenced by increased mRNA levels of these factors in astrocytes after AIT-082 administration  
5 in vitro. Moreover, since AIT-082 is orally active and rapidly passes the blood-brain barrier as shown in Example 2, it has significant therapeutic potential as an NGF-mimetic agent in Alzheimer's disease and in other neurodegenerative and cellular diseases.

10 In view of the previous results, studies were performed to demonstrate the effectiveness of using AIT-082 to treat exemplary neurodegenerative diseases. Loss of memory represents the core symptom of Alzheimer's disease as it does in a number of other neural  
15 afflictions. Specifically working (or episodic) memory is impaired in Alzheimer's disease, amnesia, aging and after hippocampal lesions in monkeys. The effects of AIT-082 in ameliorating this memory loss was used to demonstrate the efficacy of the compounds of the present invention with  
20 respect to the treatment of neurodegenerative diseases.

#### EXAMPLE 17

##### COMPARISON OF MEMORY TRACE IN DIFFERENT MICE STRAINS

The win-shift T-maze paradigm has been shown to specifically model working memory in rodents and is a  
25 widely accepted method. The rodent's natural behavior is to forage for food when hungry and therefore it will not return to the same location after it has consumed any food that was present. This model was not designed to account for all of the vast data on memory. Data from hypoxia and  
30 ischemia studies, procedures which selectively damage CA1 hippocampal cells, produce deficits in working memory while other types of memory are not affected. This strongly suggest that there are several types of memories which have different anatomical sites and most likely  
35 different neurochemical inputs. Accordingly, while the

-44-

win-shift model may not account for all neurochemical inputs involved in working memory, the model does provide a useful art accepted tool in designing pharmacological experiments to provide information on the mechanism by  
5 which memory can be modified.

Male Swiss Webster mice six months (young adult) and eleven months (old) of age, obtained from the National Institute on Aging, were maintained in individual cages, on a 22 hour light/dark cycle with continuous access to  
10 water. Food was limited so that the mice stabilized at 80% of free feeding weight. Mice were weighed and handled daily for one week. The win-shift model was run as described in the literature and consists of a T-maze in which the correct response alternates after each correct  
15 trial. The interval between trials is varied and allows for the determination of the longest period between trials that a subject can remember the correct response on the previous trial. This allows the measure of the duration of the memory trace. A score of 5 (5 correct responses  
20 per 10 trials, 50% correct) is considered chance; that is, the animal does not remember which box it selected for positive reward on the previous trial. The reward goal box is alternated after each correct trial. Ten trials per mouse are run each day. If the animal establishes a  
25 spatial learning set (right side only), they would return to the same goal each trial and have a correct response rate of significantly less than 50% correct. The latency time to leave the start box is recorded as a measure of motivation, the running time (the time from leaving the  
30 start box to reaching the goal box) is recorded as a measure of performance, and the number of correct responses as a measure of memory.

The data in Table I illustrate the effect of increasing the inter-trial interval in young adult mice  
35 without any drug treatment.

-45-

**Table I**  
*Effect of inter-trial interval in win-shift paradigm<sup>(1)</sup>*

	Inter-trial Interval (seconds)				
	30	60	90	120	150
Swiss Webster mice	7.5*	7.5*	5.0		
C57BL/6 mice	7.0*	7.4*	7.0*	7.8	5.6

- 5 <sup>(1)</sup>Score is the mean number of correct responses per 10 trials. Saline was administered 1 hour prior to testing.  
 \*p< 0.05. Data analysis following significant ANOVA, a Dunnett test was run comparing drug tested groups with controls. An Arc Sign transformation was performed on  
 10 percentage data.

From the data in Table I, it can be seen that Swiss Webster mice are capable of remembering the win-shift strategy when the inter-trial delay interval is 30 or 60 seconds. Few mice with saline treatment scored above  
 15 chance (50%) with the 90-second inter-trial delay interval. These data indicate that the "memory trace" in these animals disappears between 60 and 90 seconds. All drug evaluation tests in normal adult Swiss Webster mice were conducted with the 90-second inter-trial interval  
 20 except where indicated otherwise. In C57BL/6 mice, the duration of the memory trace was 120 seconds.

#### EXAMPLE 18

##### EFFECT OF AIT-082 ON MEMORY TRACE DURATION

The activity of AIT-082 was compared with tacrine  
 25 (THA) and physostigmine (PHY), experimental anticholinesterase agents which enhance memory in animals. The drugs were also evaluated for their effects on locomotor activity. In the win-shift memory paradigm, AIT-082 was  
 30 valuated for its ability to induce tolerance after 18 days of drug administration. In addition AIT-082 was

-46-

tested for its activity to modify learning in a modified T-maze discrimination task.

The drugs used in this example are 4-[[3-(1,6-dihydro-6-oxo-9-purin-9-yl)-1-oxopropyl]amino] benzoic acid (AIT-082), as an exemplary potassium salt, tacrine hydrochloride (tetrahydroaminoacridine, THA, Sigma Chemical Co., St. Louis, Missouri), and physostigmine, hemisulfate salt (PHY, Sigma Chemical Co., St. Louis, Missouri). The drugs were dissolved in saline and prepared fresh daily.

10 All injections were made at a volume of 0.1 ml/ 10 grams body weight. When testing drug effects, intraperitoneal (i.p.) injections of AIT-082 or THA were made one hour prior to the start of testing. Due to its shorter duration of action, PHY was injected 30 minutes prior to testing.

15 Control subjects receive a similar injection of saline (vehicle).

To determine the duration of the memory trace, subjects were administered drug or saline and 30 minutes (PHY) or 1 hour (AIT-082 or THA) later they were given a

20 single reference run with the milk reward in both goal boxes. After the indicated inter-trial delay, subjects were returned to the start box and given the first test trial with the milk reward only in the goal box opposite to the one entered on the previous correct trial. The

25 subjects were given 10 trials with the reward alternating only after correct responses.

To determine if tolerance to the biological effects of AIT-082 developed, drug or saline was administered daily for 18 days prior to the testing in the standard win-shift

30 paradigm.

Subjects were also trained in the same T-maze used for the win-shift model discussed above. As in the win-shift method, subjects were shaped and then given a single reference run in which reward was available in both goal

35 boxes. The subject was only allowed to consume the milk reward in the goal box selected. On the next run, the



-47-

reward and thus the correct response was in the same goal box selected for the reference run and was not alternated. The subject was required to learn that there was no shift in the goal box for the correct response. The subjects  
 5 were given 10 trials per day and continued until the subject had at least 8 out of 10 correct responses on two consecutive days. The number of days to reach this criteria of performance was recorded. After the subject reached criteria, the goal box for the correct response  
 10 was reversed. The number of days taken to reach criteria on reversal was recorded.

The results of the T-maze learning task and win-shift memory test are presented in Table J directly below.

Table J

15 *Effect of AIT-082, THA and PHY at 90-Second Inter-trial Interval in Swiss Webster Mice*

Type of Test <sup>(1)</sup>	Control	THA	AIT-082		PHY
	Dosage (mg/kg) Saline	1.25	0.5	30.0	0.125
Win-shift Memory Test					
20 Correct responses (Correct responses/10 trials)	4.6	7.1*	6.5*	8.2*	6.5
Latency time (seconds)	2.68	8.22*	1.95	2.03	
Running time (seconds)	1.95	3.65*	2.20	1.95	2.65
Locomotor Activity <sup>(2)</sup>	343	671*	323	378	N/T
T-maze Learning(days to reach criteria)					
25 Learning	3.6	N/T <sup>(3)</sup>	3.0	3.3	N/T
Reversal	4.2	N/T	3.78	3.5	N/T
Tolerance (Correct responses/10 trials)	4.9	N/T	N/T	7.6*	N/T

<sup>(1)</sup>at least 8 animals were run per group.

30 <sup>(2)</sup>Spontaneous movements per hour.

<sup>(3)</sup>Not tested.

\*Indicates  $p < 0.05$ . Data analysis following significant ANOVA, a Dunnett test was run comparing drug tested groups with controls. An Arc Sign transformation was performed  
 35 on percentage data and latencies were transformed to reciprocal time scores or speed scores.

-48-

As shown by the data in Table J, AIT-082 treatment resulted in an increased number of correct responses (memory) compared to saline control. While the effect was in the same range as with THA and PHY, both THA and PHY also increased latency time (prolonged the time to leave the start box, evidencing decreased motivation) and THA increased spontaneous locomotor activity. AIT-082 had no effect on learning or reversal and no tolerance developed to the memory enhancing effect of AIT-082 after 18 days of pre-treatment. Only AIT-082 enhanced memory function without affecting learning, motivation, performance and locomotor activity. Similar data have been observed with oral administration of AIT-082.

#### EXAMPLE 19

##### 15 EFFECT OF AIT-082 DOSAGE ON MEMORY TRACE DURATION

The dose response and duration of action of AIT-082 was studied in young adult Swiss Webster mice. The results are presented as the percent correct response over chance; chance being 50% correct. As shown in Fig. 7, AIT-082 is active in improving memory in normal adult Swiss Webster mice over a dose range from 0.5 to 60 mg/kg, with the optimal effect at 20 to 30 mg/kg. Further, as shown in Fig. 8, the onset of action is rapid (1 hour, data not shown) and lasts for more than seven days after a single dose of 60 mg/kg. Those skilled in the art will appreciate that the extended duration of the drug's effects will substantially lower the frequency of administration providing benefits in terms of patient compliance and cost.

## EXAMPLE 20

EFFECT OF AIT-082 ON MEMORY TRACE DURATION  
IN C57BL/6 MICE

Previous work has established that normal adult Swiss Webster mice have a memory trace duration of 60 seconds in the win-shift paradigm which may be increased by the administration of AIT-082. In order to further demonstrate the applicability and operability of the methods and compositions of the present invention, an alternative strain of mice having a different duration of memory trace was administered AIT-082, using the preceding protocol. The results are shown in Table K directly below.

Table K

Duration of Memory Trace in C57BL/6 Mice

Inter-trial interval (sec)	Treatment Groups					
	Control (Saline)		AIT-082 (30 mg/kg)		Physostigmine (0.125 mg/kg)	
	No. above chance/ Total <sup>#</sup>	Correct <sup>◇</sup>	No. above chance/ Total <sup>#</sup>	Correct <sup>◇</sup>	No. above chance/ Total <sup>#</sup>	Correct <sup>◇</sup>
30	3/5	70±11**				
60	3/5	70±16**				
90	4/5	70±6**				
120	4/5	78±16**				
150	1/5	56±10				
180	2/7	58±12	4/6	70±15**	3/6	65±16*
210			4/6	78±15**	1/6	53±9
240			0/6	50±6		
270			0/6	50±6		

<sup>#</sup> = No. subjects above chance (60% correct)/Total No. subjects tested

<sup>◇</sup> = Mean score ± S.E.

\*\* = p<0.01 (t-test against chance)

\* = p<0.05 (t-test against chance)

Typically, in the win-shift foraging paradigm, C57BL/6 mice have a duration of memory trace of 120 seconds. As

-50-

shown in Table K, at 30 mg/kg i.p., AIT-082 prolonged the duration of the memory trace to over 210 seconds. While physostigmine also prolonged the duration of the memory trace from 120 to 180 seconds in this model, it was not as  
 5 active as AIT-082.

**EXAMPLE 21****TREATMENT OF AGE INDUCED MEMORY DISORDERS USING AIT-082**

In light of the preceding results, studies were performed to demonstrate that AIT-082 improves memory in  
 10 mammals with neuronal disorders as well as in healthy subjects. Twelve-month old male Swiss Webster mice were screened for performance in the win-shift foraging test. Subjects were tested at various time delays, beginning at 10 seconds and increasing the inter-trial time interval to  
 15 30, 60, 90 and 120 seconds. The results for untreated mice are shown in Table L directly below.

**Table L**  
*Age-induced Working Memory Deficits in  
 Swiss Webster Mice*

20	Duration of Memory Trace	No. of Subjects	% of Subjects	Degree of Memory Impairment
	less than 10 seconds	6	25%	Severe
	10 seconds	8	33	Moderate
	30 seconds	<u>10</u>	<u>42</u>	Mild
25	Total	24	100	

The results in Table L demonstrate that individual subjects can be classified by the degree of working memory impairment. Subjects with severe impairment could not remember the correct response at 10 seconds while subjects  
 30 with mild deficit could remember the correct response with a 30 second inter-trial interval but not at 60 seconds. Subjects with a moderate deficit could remember the correct response with a 10 second inter-trial interval but

-51-

not at 30 seconds. Thus, the win-shift mod 1 can detect age-induced impairments in working memory. As will be appreciated by those skilled in the art, this observation is important since it provides the ability to use age-matched subjects with varying degrees of impairment for evaluation of potential therapeutic agents.

Following the establishment of a baseline, six subjects in each of the three groups were treated with AIT-082 (30 mg/kg, one hour before testing) or physostigmine (0.125 mg/kg, 30 minutes before testing) using the win-shift foraging test. The results are presented in Table M directly below and graphically represented in Fig. 9.

Table M

Effect of AIT-082 and PHY on the duration of memory trace in Swiss Webster mice with age-induced deficits

Degree of Deficit	Inter-trial Interval (sec)	Control (Saline)	AIT-082 30 mg/kg	PHY 0.125 mg/kg
Mild	60	0/6	6/6*	5/6*
	90		4/6	3/6
	120		2/6	2/6
	150		1/6	2/6
	180		1/6	1/6
	210		0/6	0/6
Moderate	30	0/6	4/6*	1/6
	60		2/6	0/6
	90		0/6	
Severe	<10	0/6	0/6	0/6

Data is presented as the number of subjects performing significantly above chance/total number of subjects;

\*Indicates  $p < 0.05$  (t-test)

Six subjects had a severe deficit with no memory trace, they could not remember the task at 10 seconds. None of these subjects showed memory restoration with

-52-

either AIT-082 or PHY treatment. In the six subjects with a moderate memory deficit who had a duration of memory trace of 10 seconds, AIT-082 increased the duration of the memory trace to greater than 30 seconds in 4 subjects (67% of the subjects) and increased the memory trace to greater than 60 seconds in two subjects (50%). In the six subjects with a mild memory deficit who had a duration of memory trace of 30 seconds, AIT-082 increased the duration of the memory trace in 2 subjects to 60 seconds, in 2 subjects to 90 seconds and in one subject each to 120 and 180 seconds. PHY increased the duration of the memory trace from 10 seconds to 30 seconds in only one animal in the moderate deficit group. In the mild deficit group, PHY increased the duration of the memory trace in 2 subjects to 60 seconds, in one subject to 90 seconds and in two subjects to at least 180 seconds. Thus, AIT-082 is more active than physostigmine in the moderate deficit group and at least as active in the mild deficit group.

#### EXAMPLE 22

##### 20 TREATMENT OF AGE DEFICIT MEMORY DISORDERS USING AIT-082

Twelve-month old male C57BL/6 mice were screened for performance in the win-shift foraging test. Subjects were tested at various inter-trial time intervals. Subjects who could not perform to criteria (>60% correct) at 10 seconds delay were classified as having a severe deficit. Subjects who performed to criteria at 10 seconds but not at 30 seconds were classified as having a moderate degree of deficit and subjects who performed to criteria at 30 seconds but not at 60 seconds were classified as having mild deficit. As in Example 21, subjects in each group were treated with either AIT-082 or PHY to determine the extent to which the working memory trace was prolonged. The results are presented in Table N directly below and graphically represented in Fig. 10.

Table N

*Effect of AIT-082 and PHY on the duration of memory trace in C57BL/6 mice with age-induced deficits*

5	Degree of Deficit	Inter-trial Interval (sec)	Control (Saline)	AIT-082 30 mg/kg	PHY 0.125 mg/kg
	Mild	60	0/6	4/4*	7/8*
		90		2/4*	3/8
		120			2/8
		150			2/8
		180			2/8
		210			0/8
	Moderate	10	6/6	6/6*	6/6
		30	0/6	4/6	1/6
		60		1/6	0/6
		90		0/6	
	Severe	<10	0/6	0/6	0/6

10 Data is presented as the number of subjects performing significantly above chance/total number of subjects; \*Indicates  $p < 0.05$  (t-test)

In the mild deficit group, AIT-082 prolonged the duration of the memory trace from 30 to 90 seconds, and from 10 to 30 seconds in the moderate deficit group. While PHY prolonged memory in the mild group, it was ineffective in the moderate group. Therefore AIT-082 restored working memory deficits in both normal mice and mice with age induced neuronal disorder for both Swiss Webster and C57BL/6 strains. Specifically, the results show that AIT-082 restores working memory in mice with mild and moderate memory deficits. Based on the other Examples previously provided it is reasonable to conclude that it accomplishes this restoration by modifying the carbon monoxide dependent guanylyl cyclase system.

-54-

**EXAMPLE 23****PROPHYLAXIS OF AGE DEFICIT MEMORY DISORDERS USING  
AIT-082**

It has been observed that age-induced memory deficits  
5 typically begin to manifest themselves in mice between 14  
and 16 months of age. Therefore, we began treating mice  
at 14 months of age with AIT-082 (30 mg/kg/day) in their  
drinking water. The animals were measured monthly for  
their memory using the win-shift foraging tests previously  
10 described. The results are shown in Fig. 11 and show that  
the administration of AIT-082 delayed the onset and  
severity of memory deficits

**EXAMPLE 24****PROPHYLAXIS OF ALCOHOL-INDUCED DEFICIT  
MEMORY DISORDERS USING AIT-82**

15

In order to demonstrate the broad applicability of the  
present invention with respect to different types of  
neurodegenerative disorders, AIT-082 was used to retard  
the production of alcohol induced memory deficit. Six  
20 month old male C57BL/6 mice were evaluated in the win-  
shift model in combination with treatment with ethanol, a  
non-specific memory suppressant, and AIT-082. Subjects  
were treated with saline (control) or AIT-082 (30 mg/kg.  
i.p.) 1 hour prior to testing. Ethanol was administered  
25 at a dose of 0.5 gm/kg i.p. ten minutes prior to testing.  
The results of a pilot study are presented in Table O  
directly below.



-55-

**Table**  
*Working memory deficit produced by ethanol and its reversal by AIT-082*

		Treatment		
		Control	Ethanol	Ethanol + AIT-082
	Correct trials <sup>1,2</sup>	8.08±0.29	6.5±26*	7.89±0.54†
5	Latency time(sec) <sup>2</sup>	1.24±0.17	1.18±0.10	1.77±0.27
	Running time(sec) <sup>2</sup>	1.44±0.35	1.17±0.08	3.22±0.61*†
	Number of subjects	13	13	9

<sup>1</sup>Indicates mean number of correct responses per 10 trials;

<sup>2</sup>Indicates mean values ±S.E.;

10 \*Indicates p<0.05 (t-test) compared to control;

†Indicates p<0.05 (t-test) compared to ethanol.

The results in Table O demonstrate that it is possible to identify a dose of a blocking agent that can produce a memory deficit as measured in the win-shift model.

15 Ethanol was selected as a non-specific blocking agent and its effects were reversed by administration of AIT-082 prior to the treatment with ethanol. Therefore it would appear feasible to evaluate other more specific blocking agents which have activity at specific receptor sites.

20 In addition to AIT-082 other purine derivatives are believed to play a role in neuronal survival, synaptogenesis and recovery of function following injury or cell death in the central nervous system. For example, similarities between guanosine and AIT-082 indicate that  
 25 AIT-082 and guanosine act through comparable mechanisms. That is, both appear to act as carbon monoxide dependent guanylyl cyclase modulators. Further, it is known that after cells are damaged, they leak massive amounts of both purine nucleosides and nucleotides to the extracellular  
 30 space. The extracellular concentration of guanosine in the region of a focal brain injury may reach 50 mM and is elevated up to five fold for at least seven days. Therefore, following injury, astrocytes or glia and

-56-

neurons are exposed to high extracellular concentrations of guanosine.

Accordingly, the following studies were undertaken in order to demonstrate the effectiveness of using other exemplary purine derivatives such as guanosine to modulate the carbon monoxide dependent guanylyl cyclase system.

#### EXAMPLE 25

##### ASTROCYTES PRODUCE TROPIC FACTORS UPON EXPOSURE TO GUANOSINE AND GTP

10 Astrocytes appear to proliferate in response to extracellular guanosine or guanosine triphosphate (GTP). GTP or guanosine may also stimulate the release of trophic factors from cultures of neocortical astrocytes from neonatal mouse brains. Astrocytes were incubated with  
15 different concentrations of guanosine or GTP respectively. Neurotrophin immunoreactivity in the culture medium from treated cells was then measured by ELISA.

Briefly, 96 well Falcon plates (Fisher) were coated with 1mg/ml of sheep mono-specific anti-NGF IgG (affinity  
20 column purified) contained in 0.1M sodium carbonate buffer pH 9.6. After an overnight incubation at 4°C blocking solution (PBS with 10% goat serum) was added to remove excess antibody. After a four hour incubation at room temperature the plates were washed 3 with PBS containing  
25 0.05% Tween 20. The conditioned media and standard 2.5S HPLC purified NGF were added and incubated overnight. The next day plates were washed 3 times with PBS-0.05% Tween 20. The secondary antibody, rabbit mono-specific anti-NGF IgG conjugated with b-galactosidase (Pierce-SPDP  
30 method) (1:500 dilution) was added. The plates were incubated overnight at 4°C. The next day the plates were washed 3 times with PBS-0.05% Tween 20. To each well substrate, 0.2mM 4-methylumbelliferyl-b-galact side (MUG) in 0.1M phosphate buffer (1mM MgCl<sub>2</sub>, pH 7.2) was added.

-57-

After a 4 hour incubation at room temperature the reaction was stopped by the addition of 0.1M glycine, pH 10.3. Samples were then read using Microfluor ELISA reader (excitation 360 nm; emission 450nm). The sensitivity of this assay was 10pg/well NGF.

The ELISA assay detected neurotrophins NGF and NT-3 with almost equal affinity and BDNF with 100 times less affinity. As shown in Figs. 12A and 12B, both guanosine and GTP increased the amount of NGF-like immunoreactivity in the culture medium. The astrocytes exposed to the various levels of guanosine produced a much stronger response than those exposed to equivalent concentrations of GTP.

#### EXAMPLE 26

##### ASTROCYTES PRODUCE NEUROTROPHIC FACTORS UPON EXPOSURE TO GUANOSINE

In order to confirm the results of the previous assay, mRNA levels of the tropic factors FGF-2 and NGF were measured in astrocytes which had been exposed to guanosine. The mRNA levels were measured using the same protocol used previously in Example 16. As shown in Figs. 13A and 13B, the addition of guanosine increased NGF and FGF-2 mRNA at 4 hours and at 24 hours, respectively, after it was added to astrocytes. The observed increase in neurotrophin mRNA is important following brain injury or recovery from brain injury when the extracellular concentration of guanosine is considerably high. As cells are exposed to a high concentration of guanosine for several days following brain injury, this data indicates that guanosine may be responsible for some of the recovery of function.

As previously discussed, an agent that can penetrate the blood brain barrier and increase concentrations of neurotrophic factors as measured here by mRNA levels should have a substantial positive effect on neuronal survival and on the formation of collateral nerve cir-

cuits. In turn, this should enhance functional recovery in many different neurological diseases or after damage to the nervous system.

### EXAMPLE 27

**NEURONS UNDERGO NEURITOGENESIS UPON EXPOSURE TO GUANOSINE**

In addition to changes in glia or astrocytes, important neuronal changes also take place following focal brain injury. Neuritic processes of surviving neurons may undergo neuritogenesis. Accordingly, based on previous results using AIT-082, studies were performed to demonstrate that guanosine may also modify carbon monoxide guanylyl cyclase to stimulate neuritogenesis. As previously discussed, because PC12 cells constitute a homogeneous population of neuronal-like cells, without contaminating astroglia-type cells, the direct effects of the exemplary purine derivatives of the present invention on neurite outgrowth in these cells can be observed easily. Accordingly, PC12 cells were exposed to guanosine and adenosine with and without NGF and monitored as in Example 12. The effects of exposure to purine derivatives with NGF are shown in Fig. 14A while exposure without NGF is shown in Fig. 14B. A direct comparison of the effects of these purine derivatives with and without the presence of NGF is shown for each compound in Fig. 14C.

As shown in Fig. 14A, guanosine, but not adenosine, enhanced the neurite outgrowth induced by NGF in PC12 cells after 48 hours. The enhancement was significant over that of NGF alone at guanosine concentrations of 30 and 300 mM. Adenosine did not enhance NGF induced neurite outgrowth at any concentration. This indicates that neurite outgrowth induced by purines is not just a generalized phenomenon. 5'-N-ethylcarboxamidoadenosine (NECA), an adenosine A<sub>1</sub> and A<sub>2</sub> receptor agonist, also enhanced neurite outgrowth, but not to the same extent as guanosine.

-59-

On their own, in the absence of NGF, both adenosine and guanosine slightly increased the proportion of cells with neurites as shown in Fig. 14B. The effects of guanosine at both 30 and 300 mM was greater than adenosine at the same concentrations. In the presence of (NECA), there was little stimulation of neurite outgrowth. Because the effects of the compounds in the presence of NGF were much more readily scored and less variable from experiment to experiment than with the compounds alone, most of the data for enhancement of neurite outgrowth was determined in the presence of NGF.

The comparative data shown in Figs. 14A and 14B and emphasized in Fig. 14C show that guanosine causes some neurite extension, but can also react synergistically to enhance the trophic effects of NGF. Adenosine, although slightly enhancing neurite outgrowth on its own does not enhance the effects of NGF. Interestingly, NECA but not adenosine could synergistically enhance the actions of guanosine, both in the presence and absence of NGF as shown in Fig. 14C. The fact that adenosine did not increase NGF-dependent neurite outgrowth in PC12 cells but that guanosine did, suggests that they interact differently with PC12 cells. Adenosine would interact with adenosine receptors, such as the  $A_2$  purinoceptor. This would activate adenylate cyclase which increases intracellular cAMP. NECA apparently acts in this manner. But the effects of NECA were synergistic with those of guanosine. This indicates that guanosine and NECA use different signalling pathways to enhance neurite outgrowth.

#### EXAMPLE 28

##### VARIOUS PURINE DERIVATIVES

##### PROVIDE DIFFERENT RATES OF NEURITE GROWTH

In view of the previous results, other exemplary purine derivatives were examined to demonstrate the

-60-

specificity of those compounds which modulate carbon monoxide dependent guanylyl cyclase to modify neural activity. Specifically, different concentrations of the purine derivatives inosine, hypoxanthine and xanthine were  
5 tested in the presence of NGF using the protocol of Example 12 to demonstrate their ability to modify neural activity.

As shown in Fig. 15A, inosine only slightly enhanced neurite outgrowth over that produced in cells treated with  
10 NGF alone. This was true for concentrations of inosine ranging from 0.3 to 300 mM. Fig. 15A also shows that the action of inosine on the enhancement of neurite outgrowth was much less effective than that of guanosine.

Figs. 15B and 15C also show that hypoxanthine and  
15 xanthine each produced results similar to that of inosine on NGF-induced neuritogenesis. In Fig. 15C xanthine, in concentrations from 0.3 to 30 mM (300 mM was toxic to the cells), only slightly enhanced NGF-induced neurite outgrowth. Fig. 15B shows that hypoxanthine showed the  
20 greatest, although still modest, enhancement at concentrations of 0.3 and 300 mM, although other concentrations had no significant enhancement. Even though some enhancement of neurite outgrowth was observed with hypoxanthine, the relative amount of enhancement was not nearly as great as  
25 was the effect of guanosine. These results indicate that inosine, xanthine and hypoxanthine do not modulate the carbon monoxide-dependent guanylyl cyclase system to modify neural activity but rather influence other signaling mechanisms.

30

#### EXAMPLE 29

##### EFFECTS OF AIT-34 ON NEURITOGENESIS

To demonstrate the effects of compounds similar to AIT-082 on neuritogenesis, PC12 cells were exposed to AIT-34, otherwise known as 3(1,6 dihydro-6- xo-9h purin-9-  
35 yl)-N-[3-(2-oxopyrrolidin-1-yl) propyl] propanamide,

-61-

during growth and monitored according to Example 12. As shown in Fig. 16, different concentrations of AIT-034 did not enhance NGF-induced neuritogenesis as is observed with AIT-082.

5

**EXAMPLE 30****EFFECTS OF ATP AND GTP ON NEURITOGENESIS**

To further demonstrate that purine derivatives having different functional groups may be used in accordance with the teachings of the present invention, PC12 cells were  
10 exposed to adenosine triphosphate (ATP) and guanosine triphosphate (GTP) and monitored for neuritogenesis using the protocol of Example 12.

In a manner very similar to the actions of adenosine and guanosine on neurite outgrowth in PC12 cells, their  
15 corresponding nucleotides ATP and GTP had parallel effects on neurite outgrowth. As shown in Fig. 17, ATP did not enhance neuritogenesis in either NGF treated cells or on its own. In sharp contrast, GTP at 30 and 300 mM, did enhance neuritogenesis in the presence of NGF and further  
20 elicited neurite outgrowth on its own.

However, as shown in Fig.18, GTP did not appear to be acting as a source from which guanosine was released in a controlled manner. If GTP was hydrolyzed to guanosine diphosphate (GDP), guanosine monophosphate (GMP) and finally  
25 to guanosine by ectoenzymes, one would predict that GDP and GMP would also enhance neurite outgrowth from PC12 cells. Yet, neither GDP nor GMP were effective alone or with NGF in eliciting neurite outgrowth. By way of comparison, the adenine-based compounds all had an inhibitory  
30 effect.

**EXAMPLE 31****GUANOSINE BUT NOT GTP INCREASES cGMP IN PC12 CELLS**

Based on the previous examples, a study was conducted to demonstrate the neurotogenic mechanisms of GTP and

-62-

guanosine respectively. Guanosine and GTP have been shown to increase intracellular cyclic 3',5'-guanosine monophosphate (cGMP) in arterial smooth muscle. Since cGMP analogues have been reported to stimulate neurite outgrowth from neuroblastoma cells it was possible that both guanosine and GTP might exert their effects through increasing intracellular cGMP. As shown in Fig. 19, guanosine increased intracellular cGMP in PC12 cells as determined by radioimmunoassay using the protocol detailed in Example 15. Such an increase would be expected of a carbon monoxide dependent guanylyl cyclase modulator. In contrast, it was found that GTP did not increase levels of cGMP, indicating that any GTP-stimulated neuritogenesis occurs by another mechanism.

15

**EXAMPLE 32****USE OF NON-SELECTIVE INHIBITORS OF GUANYLYL CYCLASE REDUCES GUANOSINE NEURITOGENESIS**

To demonstrate that guanosine modifies the carbon monoxide-dependent guanylyl cyclase system, studies were conducted to show that increased levels of intracellular cGMP were necessary for guanosine to enhance NGF's neurotogenic effects on PC12 cells. In particular, different concentrations of three inhibitors of guanylyl cyclase were added to PC12 cells with guanosine. Neuritogenesis was then determined using the protocol of Example 12.

Methylene Blue (MB) inhibits soluble guanylyl cyclase (sGC), the enzyme that synthesizes cGMP. As shown in Fig. 20A the addition of MB (0.1 - 5 mM) to cultures of PC12 cells abolished the synergistic effects of guanosine with NGF. Conversely, MB had no effect on NGF-stimulated neurite outgrowth.

LY83583 inhibits both particulate and sGC. Fig. 20B shows that the neurite outgrowth response elicited by guanosin was inhibited by LY83583, but the response elicited by NGF was unaffected. The mechanism by which



-63-

LY83583 inhibits guanylyl cyclase is unresolved, but is likely indirect, involving glutathione metabolism. Therefore, two non-selective inhibitors of guanylyl cyclase, each with a different mechanism of action, attenuated the neurotogenic action of guanosine.

These data indicate that guanosine and NGF act through different mechanisms. They also indicate that increases in intracellular cGMP were necessary, although possibly not sufficient, for guanosine to exert its neurotogenic effects.

To test whether increases in cGMP were sufficient to cause neurite outgrowth, atrial natriuretic factor (ANF) was added to cell cultures in a manner similar to that used for guanosine. ANF is a hormone whose only known signal transduction pathway is through activation of particulate guanylyl cyclase. As shown in Fig. 20C, ANF, like guanosine, enhanced NGF-stimulated neurite outgrowth from PC12 cells indicating that increased intracellular cGMP production, induced by carbon monoxide dependent guanylyl cyclase or other mechanisms assisted in stimulating neurite outgrowth.

### EXAMPLE 33

#### NITRIC OXIDE OR CARBON MONOXIDE PROMOTES GUANOSINE NEURITOGENESIS

Because guanosine increased intracellular cGMP as shown in Example 31, studies were performed to demonstrate whether its signal could be transduced through production of NO or CO. If NO was involved, then addition of nitric oxide donors that liberate NO should mimic the effects of guanosine.

PC12 cells were grown for 48 hours in the presence of sodium nitroprusside (SNP) or sodium nitrite (SN), both of which liberate NO. Alone, neither SNP nor SN elicited neurite outgrowth from PC12 cells. However, like guanosine, both SNP and SN enhanced NGF-mediated neurite out-

-64-

growth in a synergistic manner as shown for the addition of SN in Fig. 21. Further confirming the effect, Figs. 22A and 22B show that the neurotogenic properties of the NO donors were inhibited by both hemoglobin (Hb) and methemoglobin (MB). Both are substances which scavenge NO and CO with high affinity and preclude these agents from being used as signal transmitters.

Accordingly, if NO or CO mediates the neurotogenic effects of guanosine, then these effects should be reduced by addition of hemoglobin to the cultures. The expected effect is clearly shown in Fig. 23 where Hb (0.1-1 mM) inhibited the neurotogenic effects of guanosine but not those of NGF. This indicates that the neurotogenic action of guanosine, but not that of NGF, requires synthesis of NO or CO.

Several facts indicate that it is CO rather than NO which interacts with guanosine to modify neural activity. For example, if the effects of guanosine were mediated through NO, then addition of guanosine to the PC12 cells should stimulate cNOS in PC12 cells to produce NO. However, cNOS had not been reported in PC12 cells and untreated (guanosine and NGF naive) PC12 cells did not stain for diaphorase, an enzyme that co-localizes with NOS. Since cNOS is calcium/calmodulin-sensitive, its activity should increase after adding a calcium ionophore, thus leading to increased cGMP levels. Addition of the ionophore A23187 to cultures of PC12 cells failed to elicit an increase in cGMP.

#### EXAMPLE 34

##### **CARBON MONOXIDE, NOT NITRIC OXIDE, MEDIATES THE EFFECTS OF GUANOSINE ON NEURITOGENESIS**

Based on the results of the previous examples, studies were performed to demonstrate that the purine derivatives of the present invention, including guanosine, modulate

-65-

the carbon monoxide-dependent guanylyl cyclase system to modify neural activities.

As in Example 6 where it was shown that carbon monoxide mediates the effects of AIT-082 through the use of inhibitors, the same techniques demonstrate that guanosine also interacts with the carbon monoxide dependent system. Specifically, as shown in Fig. 24, the cNOS inhibitor L-nitro arginine methyl ester (L-NAME) did not affect the ability of guanosine to enhance NGF-mediated neurite outgrowth. These data confirm that cNOS was not involved in the signal transduction pathway that mediated the neurotogenic effects of guanosine on PC12 cells.

To further demonstrate that CO, rather than NO, mediated the neurotogenic effects of guanosine, zinc protoporphyrin IX (ZnPP), which inhibits heme oxygenase and hence inhibits CO synthesis, was added to the cells during growth. As shown in Fig. 25, ZnPP abolished the neurotogenic effects of guanosine, but did not affect those of NGF. In contrast, a related protoporphyrin derivative, copper protoporphyrin IX (CuPP), does not inhibit heme oxygenase. Accordingly, Fig. 26 shows that copper protoporphyrin IX did not reduce the ability of guanosine to enhance NGF-dependent neurite outgrowth from PC12 cells. As with AIT-082, these data indicate that guanosine increased CO synthesis. In turn, CO activated sGC and increased intracellular GMP, thereby promoting neuritogenesis.

### EXAMPLE 35

#### INOSINE PRANOBEX ENHANCES NEURITOGENESIS

To provide further evidence of the scope and operability of the present invention, neurotogenic studies were performed using inosine pranobex. Specifically, inosine pranobex is a mixture of inosine and DIP-PacBa at a 1:3 molar ratio. Various concentrations of this compound were

-66-

added to PC12 cells with NGF which were then monitored according to the protocol of Example 12.

As shown in Fig. 27, inosine pranobex substantially enhanced the amount of neurite outgrowth of the treated cells. The curve shown in Fig. 27 represents the different levels of inosine pranobex plus saturating concentrations of NGF while the horizontal lines represent the NGF control with attendant confidence levels. Here the treated cells are above the control baseline at most of the selected concentrations.

The modification of cellular and, more specifically, neural activity in accordance with the teachings of the present invention may be used to treat a wide variety of cellular and neurodegenerative diseases in order to provide recovery of cellular or neural function. Thus, the present invention may be used to treat cellular and neurodegeneration from any cause including oxidative stress, disease, trauma, age, and exposure to harmful physical or chemical agents. Similarly, the methods and medicaments disclosed herein may be used to treat neurological diseases including, but not limited to, Alzheimer's Disease and related degenerative disorders, Parkinson's disease and related disorders such as striatonigral degeneration, spino-cerebellar atrophies, motor neuronopathies or "motor system diseases" including Amyotrophic Lateral Sclerosis, Werdnig-Hoffmann disease, Wohlfart-Kugelberg-Welander syndrome and hereditary spastic diplegia, damage to neurons by ischemia (as in strokes), anoxia, or hypoglycemia (as, for example, after prolonged circulatory arrest), Huntington's disease, cerebral palsy, multiple sclerosis, psychiatric disorders including affective disorders, schizophrenia, epilepsy and seizures, peripheral neuropathies from any cause, learning disabilities and disorders of memory. Also, damage to neurons or their processes by physical agents such as

-67-

radiation or electrical currents or by chemical agents including alcohol, aluminum, heavy metals, industrial toxins, natural toxins and legal or illegal drugs may be treated. The methods may further be used to treat victims  
5 of trauma to the brain or spinal cord resulting in neuronal damage or age related conditions such as benign forgetfulness and deterioration of sensory, motor, reflex or cognitive abilities due to loss of neurons or neuronal connectivity. Simply administering an effective dosage of  
10 at least one of the carbon monoxide dependent guanylyl cyclase modulating purine derivatives of the present invention to a subject suffering from any of the foregoing cellular or neural disorders will induce intracellular changes producing restoration of function.

15 Specifically, modification of the carbon monoxide dependent guanylyl cyclase system in accordance with the teachings of the present invention produces changes in neural activity in neurons and glia cells including astrocytes. For example, using the present invention the  
20 neural activity of astrocytes may be modified to synthesize various neurotrophic factors and cytokines including fibroblast growth factor (FGF), nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). These factors can influence the  
25 sprouting of neuritic processes from surviving neurons as well as promote the development of new cells. New synapses may then form and provide some recovery of function. These neurotrophic factors also play a neuroprotective role. Thus, inducing their production can  
30 ameliorate further neural damage.

Numerous purine derivatives may be used in accordance with the teachings of the present invention. However, the ability to modify neural activity by modulating the carbon monoxide dependent guanylyl cyclase system is not a  
35 general property of all purines or purine derivatives. For example, as shown in the data below, inosine,

aden sine, hyp xanthin and xanthine were all relatively ineffective at modifying neural activity. Other purine derivatives which failed to modify neural activity include 3-(6-amino-9H-purin-9-yl)propionic acid, ethyl ester (AIT-0026), 3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-N-{3-(2-oxopyrrolidin-1-yl)propyl}propanamide (AIT-0034) and propentofylline. Moreover, while other purines and purine derivatives such as 5'-N-ethylcarboxamidoadenosine (NECA) were shown to stimulate neurite outgrowth, they did not do so by modulation of the carbon monoxide dependent guanylyl cyclase mechanism. Accordingly, the scope of the invention is defined by the functional reactivity of purine derivatives which modify cellular or neural activity as described herein and as shown by the data presented. Of course, those skilled in the art will appreciate that functionally equivalent isomers, analogs and homologs of the compounds of the present invention may be substituted.

Those skilled in the art will further appreciate that the present invention may be embodied in other specific forms without departing from the spirit or central attributes thereof. In that the foregoing description of the present invention discloses only exemplary embodiments thereof, it is to be understood that other variations are contemplated as being within the scope of the present invention. Accordingly, the present invention is not limited to the particular embodiments which have been described in detail herein. Rather, reference should be made to the appended claims as indicative of the scope and content of the present invention.

## WHAT IS CLAIMED IS:

1. A medicament for the long term modification of mammalian neural activity, said medicament comprising an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.
2. The medicament of claim 1 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxo-  
5 propyl]amino]benzoic acid, and inosine pranobex.
3. The medicament of claim 1 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.
4. The medicament of claim 2 configured for orally administering said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative to a mammalian subject.
5. The medicament of claim 2 configured for injecting said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative into a mammalian subject.
6. The medicament of claim 1 wherein said modified mammalian neural activity is the production of neurotrophins.
7. The medicament of claim 1 wherein said modified mammalian neural activity is the production of pleiotrophins.

8. The medicament of claim 1 wherein said modified mammalian neural activity is enhanced neuronal survival.

9. The medicament of claim 1 wherein said modified mammalian neural activity is the formation of collateral nerve circuits.

10. The medicament of claim 1 wherein said modified mammalian neural activity is neuritogenesis.

11. The medicament of claim 1 wherein said modified mammalian neural activity is synapse formation.

12. The medicament of claim 1 wherein said modified mammalian neural activity is the production of cyclic purine nucleotides.

13. The medicament of claim 1 wherein said modified mammalian neural activity is enhanced neurotrophin or pleiotrophin activity.

14. The medicament of claim 13 wherein said enhanced neurotrophin or pleiotrophin activity is produced by a neurotrophic factor selected from the group consisting of nerve growth factor, fibroblast growth factor,  
5 neurotrophin-3, brain derived neurotrophic factor, neurotrophin-4/5 ciliary neurotrophic factor, S100B and combinations thereof.

15. A medicament for the treatment of mammalian neurological diseases comprising an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

16. The medicament of claim 15 wherein said carbon monoxide dependent guanylyl cyclase modulating purine



-71-

derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxo-5 propyl]amino]benzoic acid, and inosine pranobex.

17. The medicament of claim 15 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.

18. The medicament of claim 16 configured for orally administering said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative to said mammalian subject.

19. The medicament of claim 16 configured for injecting said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative in said mammalian subject.

20. The medicament of claim 15 wherein said mammalian neurological disease is Alzheimer's disease and related degenerative disorders.

21. The medicament of claim 15 wherein said mammalian neurological disease is old age benign forgetfulness and related disorders.

22. The medicament of claim 15 wherein said mammalian neurological disease is aging related loss of neurons or neuronal connectivity and related deterioration of sensory, motor, reflex, or cognitive abilities.

23. The medicament of claim 15 wherein said mammalian neurological disease is Parkinson's disease and related disorders.

-72-

24. The medicament of claim 15 wherein said mammalian neurological disease is spino-cerebellar atrophy.

25. The medicament of claim 15 wherein said mammalian neurological disease is motor neuronopathy.

26. The medicament of claim 15 wherein said mammalian neurological disease is damage to neurons or their processes by physical agents.

27. The medicament of claim 15 wherein said mammalian neurological disease is damage to neurons by ischemia, anoxia, or hypoglycemia.

28. The medicament of claim 15 wherein said mammalian neurological disease is damage to neurons by chemical agents.

29. The medicament of claim 15 wherein said mammalian neurological disease is trauma to the brain or spinal cord.

30. The medicament of claim 15 wherein said mammalian neurological disease is epilepsy or seizures.

31. The medicament of claim 15 wherein said mammalian neurological disease is peripheral neuropathy.

32. The medicament of claim 15 wherein said mammalian neurological disease is learning disability.

33. The medicament of claim 15 wherein said mammalian neurological disease is cerebral palsy.

34. The medicament of claim 15 wherein said mammalian neurological disease is psychiatric disorder.

35. The medicament of claim 15 wherein said mammalian neurological disease is memory disorder.

36. The medicament of claim 15 wherein said mammalian neurological disease is Huntington's disease.

37. A medicament for inducing long term changes in the membrane potential of a mammalian neuron, said medicament comprising an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating  
5 purine derivative.

38. The medicament of claim 37 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxopropyl]amino]benzoic acid, and inosine pranobex.  
5

39. The medicament of claim 37 wherein said effective amount of said at least one carbon monoxide-dependent, guanylyl cyclase modulating, purine derivative produces a treating concentration of at least 1  $\mu$ M.

40. A medicament for selectively and controllably inducing the *in vivo* genetic expression of at least one naturally occurring genetically encoded molecule in a mammal, said medicament comprising an effective amount of  
5 at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

41. The medicament of claim 40 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxopropyl]amino]benzoic acid, and inosine pranobex.  
5

42. The medicament of claim 40 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.

43. The medicament of claim 40 wherein said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative is orally administered to said mammal.

44. The medicament of claim 40 wherein said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative is administered to said mammal by injection.

45. The medicament of claim 40 wherein said at least one naturally occurring genetically encoded molecule is a neurotrophic factor.

46. The medicament of claim 45 wherein said neurotrophic factor is a neurotrophin.

47. The medicament of claim 45 wherein said neurotrophic factor is a pleiotrophin.

48. The medicament of claim 45 wherein said neurotrophic factor is selected from the group consisting of nerve growth factor, fibroblast growth factor, neurotrophin-3, brain derived neurotrophic factor,  
5 neurotrophin-4/5 ciliary neurotrophic factor, S100B and combinations thereof.

49. A medicament for the effective direct administration of at least one naturally occurring genetically encoded molecule in a mammal by selectively inducing the *in vivo* genetic expression of said molecule

-75-

- 5 in said mammal, said medicament comprising the step of an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative to said mammal.

50. The medicament of claim 49 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxo-propyl]amino]benzoic acid, and inosine pranobex.

51. The medicament of claim 49 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.

52. The medicament of claim 49 wherein said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative is orally administered to said mammal.

53. The medicament of claim 49 wherein said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative is administered to said mammal by injection.

54. The medicament of claim 49 wherein said at least one naturally occurring genetically encoded molecule is a neurotrophic factor.

55. The medicament of claim 54 wherein said neurotrophic factor is a neurotrophin.

56. The medicament of claim 54 wherein said neurotrophic factor is a pleiotrophin.

57. The medicament of claim 54 wherein said neurotrophic factor is selected from the group consisting of nerve growth factor, fibroblast growth factor, neurotrophin-3, brain derived neurotrophic factor, 5 neurotrophin-4/5 ciliary neurotrophic factor, S100B and combinations thereof.

58. A medicament for the treatment of mammalian disease conditions associated with cellular damage due to oxidative stress by inducing the *in vivo* production of at 10 least one naturally occurring endogenous antioxidant, said medicament comprising the step of an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

59. The medicament of claim 58 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxo- 5 propyl]amino]benzoic acid, and inosine pranobex.

60. The medicament of claim 58 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.

61. The medicament of claim 59 configured for orally administering said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

62. The medicament of claim 59 configured for injecting said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

-77-

63. The medicament of claim 58 wherein said mammalian disease condition is Alzheimer's disease and related degenerative disorders.

64. The medicament of claim 58 wherein said mammalian disease condition is old age benign forgetfulness and related disorders.

65. The medicament of claim 58 wherein said mammalian disease condition is aging related loss of neurons or neuronal connectivity and related deterioration of sensory, motor, reflex, or cognitive abilities.

66. The medicament of claim 58 wherein said mammalian disease condition is Parkinson's disease and related disorders.

67. The medicament of claim 58 wherein said mammalian disease condition is spino-cerebellar atrophy.

68. The medicament of claim 58 wherein said mammalian disease condition is motor neuronopathy or Amyotrophic Lateral Sclerosis.

69. The medicament of claim 58 wherein said mammalian disease condition is damage to neurons or their processes by physical agents.

70. The medicament of claim 58 wherein said mammalian disease condition is damage to neurons by ischemia, anoxia, hypoxia, or hypoglycemia.

71. The medicament of claim 58 wherein said mammalian disease condition is damage to neurons by chemical agents.

-78-

72. The medicament of claim 58 wherein said mammalian disease condition is trauma to the heart, brain or spinal cord.

73. The medicament of claim 58 wherein said mammalian disease condition is epilepsy or seizures.

74. The medicament of claim 58 wherein said mammalian disease condition is peripheral neuropathy.

75. The medicament of claim 58 wherein said mammalian disease condition is learning disability.

76. The medicament of claim 58 wherein said mammalian disease condition is cerebral palsy.

77. The medicament of claim 58 wherein said mammalian disease condition is psychiatric disorder.

78. The medicament of claim 58 wherein said mammalian disease condition is memory disorder.

79. The medicament of claim 58 wherein said mammalian disease condition is Huntington's disease.

80. The medicament of claim 58 wherein said endogenous antioxidant is a bile pigment.

81. The medicament of claim 80 wherein said bile pigment is selected from the group consisting of biliverdin or bilirubin.

82. The medicament of claim 80 wherein said bile pigment is produced through the step of degrading heme with heme oxygenase.



-79-

83. A medicament for inducing the *in vivo* production of heme oxygenase in a mammal, said medicament comprising an effective amount of at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

84. The medicament of claim 83 wherein said carbon monoxide dependent guanylyl cyclase modulating purine derivative is selected from the group consisting of guanosine, 4-[[3-(1,6-dihydro-6-oxo-9H-purin-9-yl)-1-oxo-  
5 propyl]amino]benzoic acid, and inosine pranobex.

85. The medicament of claim 83 wherein said effective amount of said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative produces a treating concentration of at least 1  $\mu$ M.

86. The medicament of claim 84 configured for orally administering said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

87. The medicament of claim 84 configured for injecting said at least one carbon monoxide dependent guanylyl cyclase modulating purine derivative.

88. The medicament of claim 83 induces the degrading of heme in said mammal with said heme oxygenase to endogenously produce bile pigment and carbon monoxide.

89. The medicament of claim 88 wherein said medicament modulates guanylyl cyclase in said mammal with said endogenously produced carbon monoxide.

90. The medicament of claim 88 wherein said medicament neutralizes or sequesters free radicals in said mammal with said endogenously produced bile pigment.

-80-

91. The medicament of claim 88 wherein said medicament induces a reduction in the blood pressure of said mammal with said endogenously produced carbon monoxide.

1/21

FIG. 1.

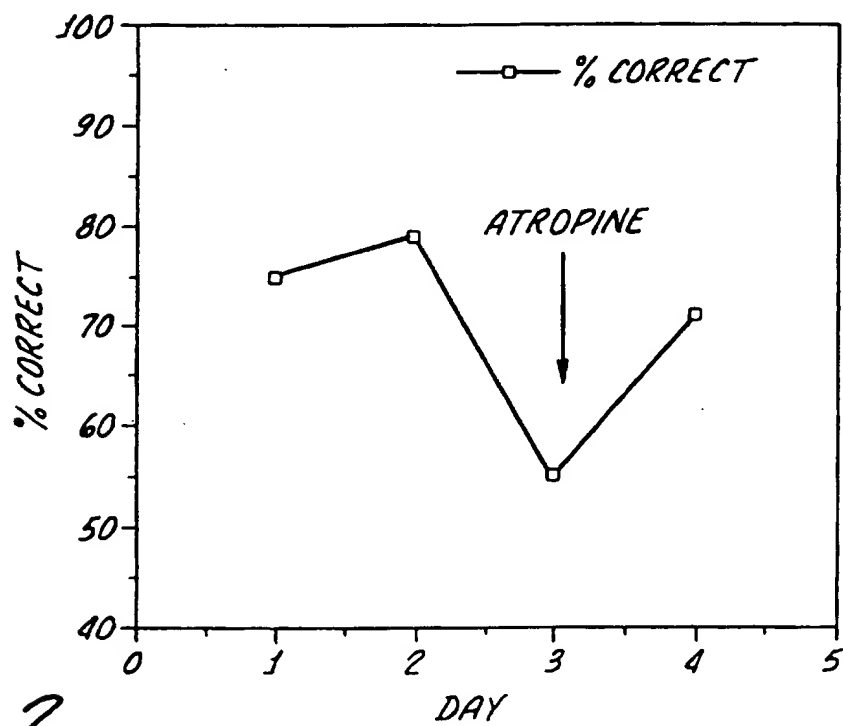
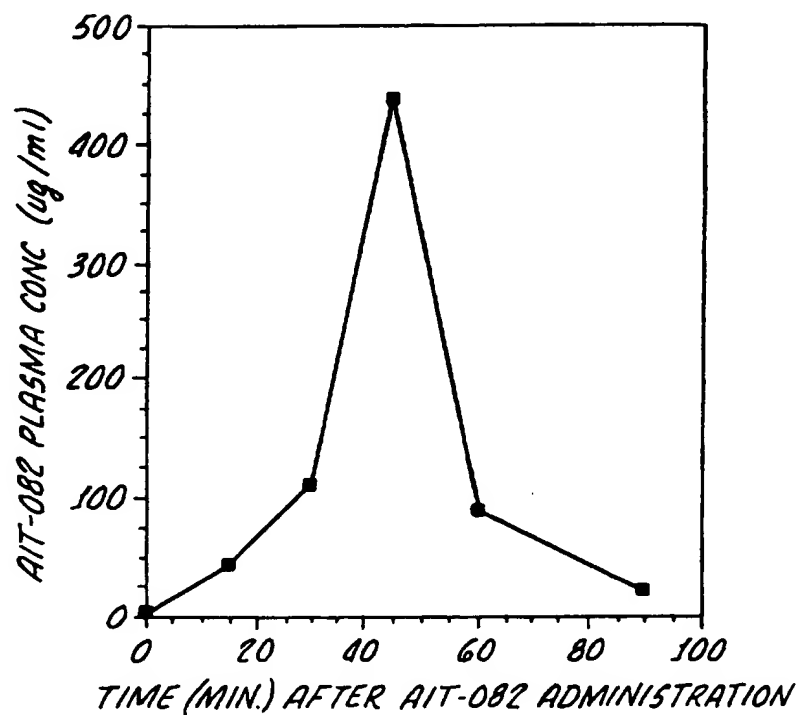


FIG. 2.

2/21

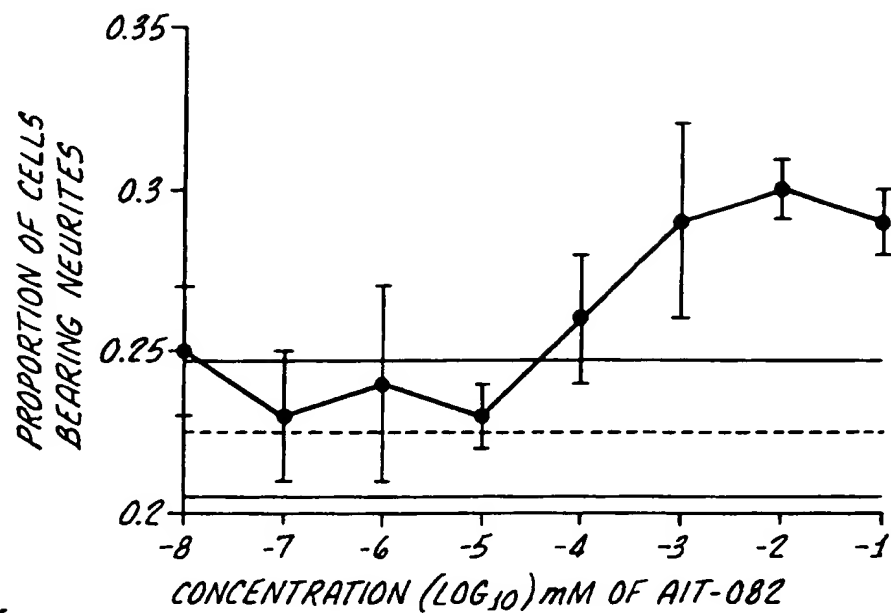


FIG. 3.

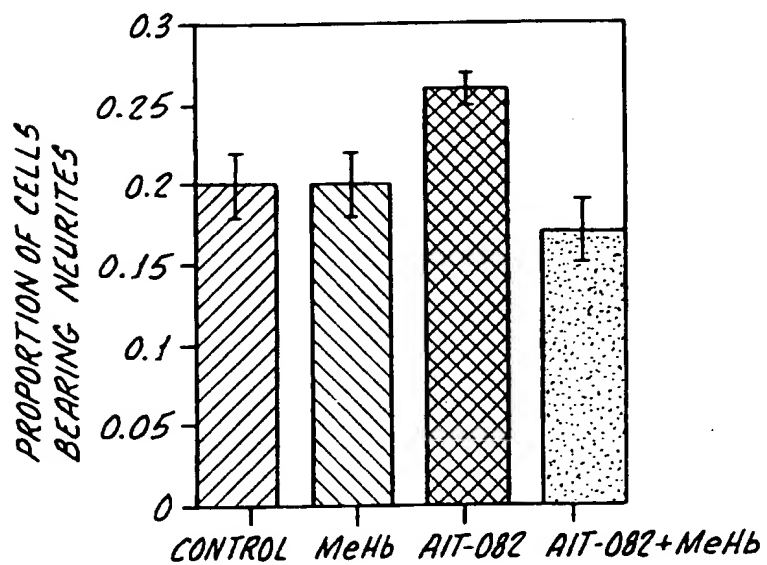


FIG. 4A.

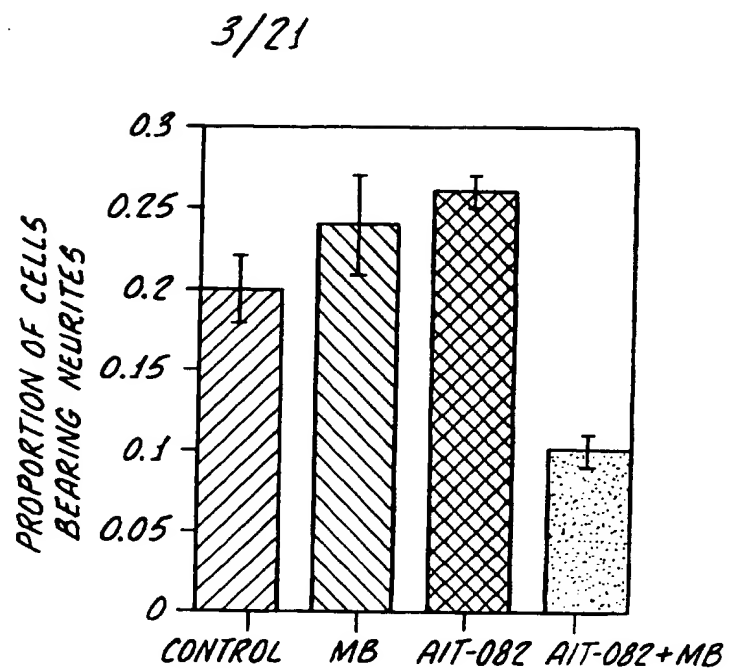


FIG. 4B.

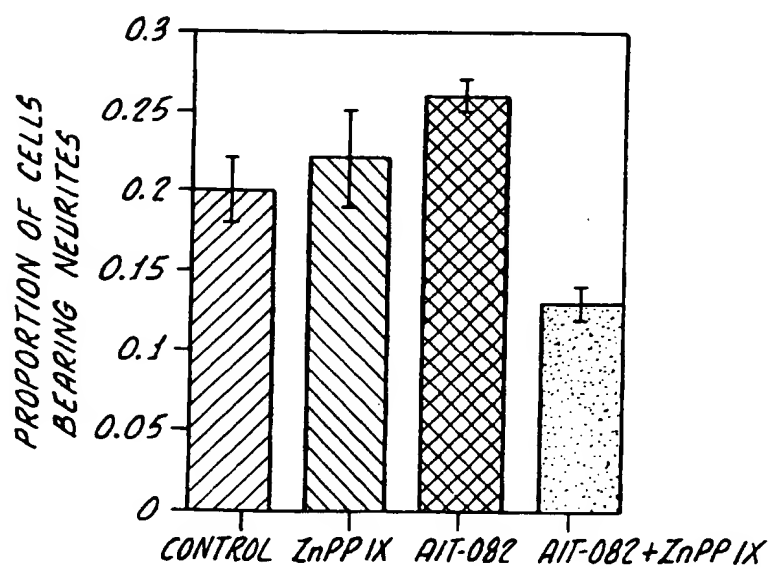
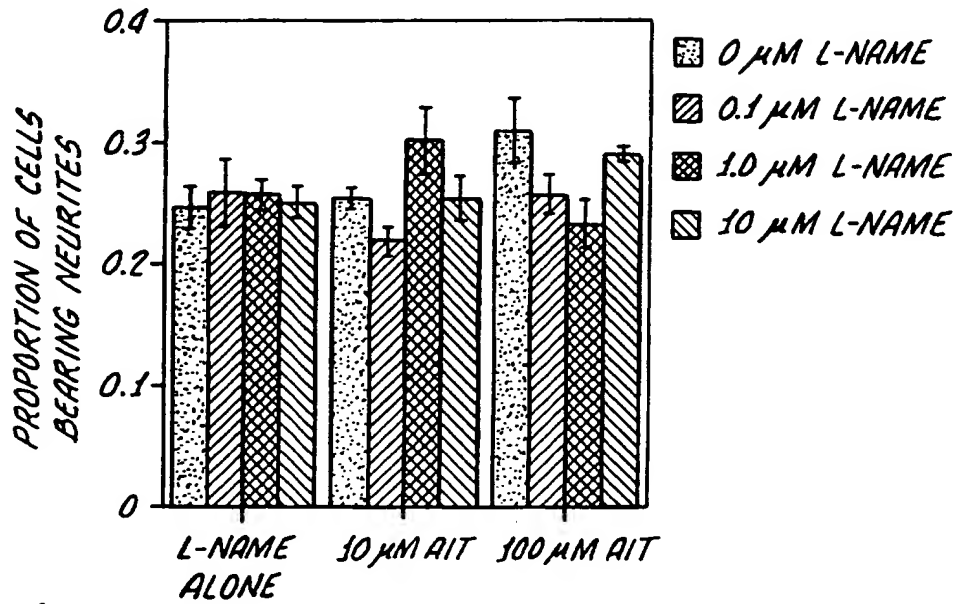
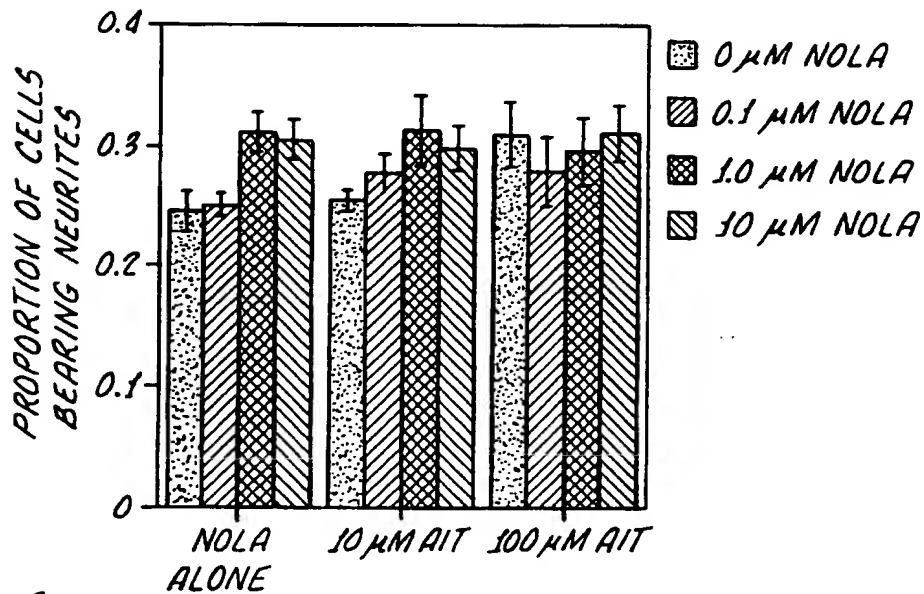


FIG. 4C.

4/21

FIG. 5A.FIG. 5B.

5/21

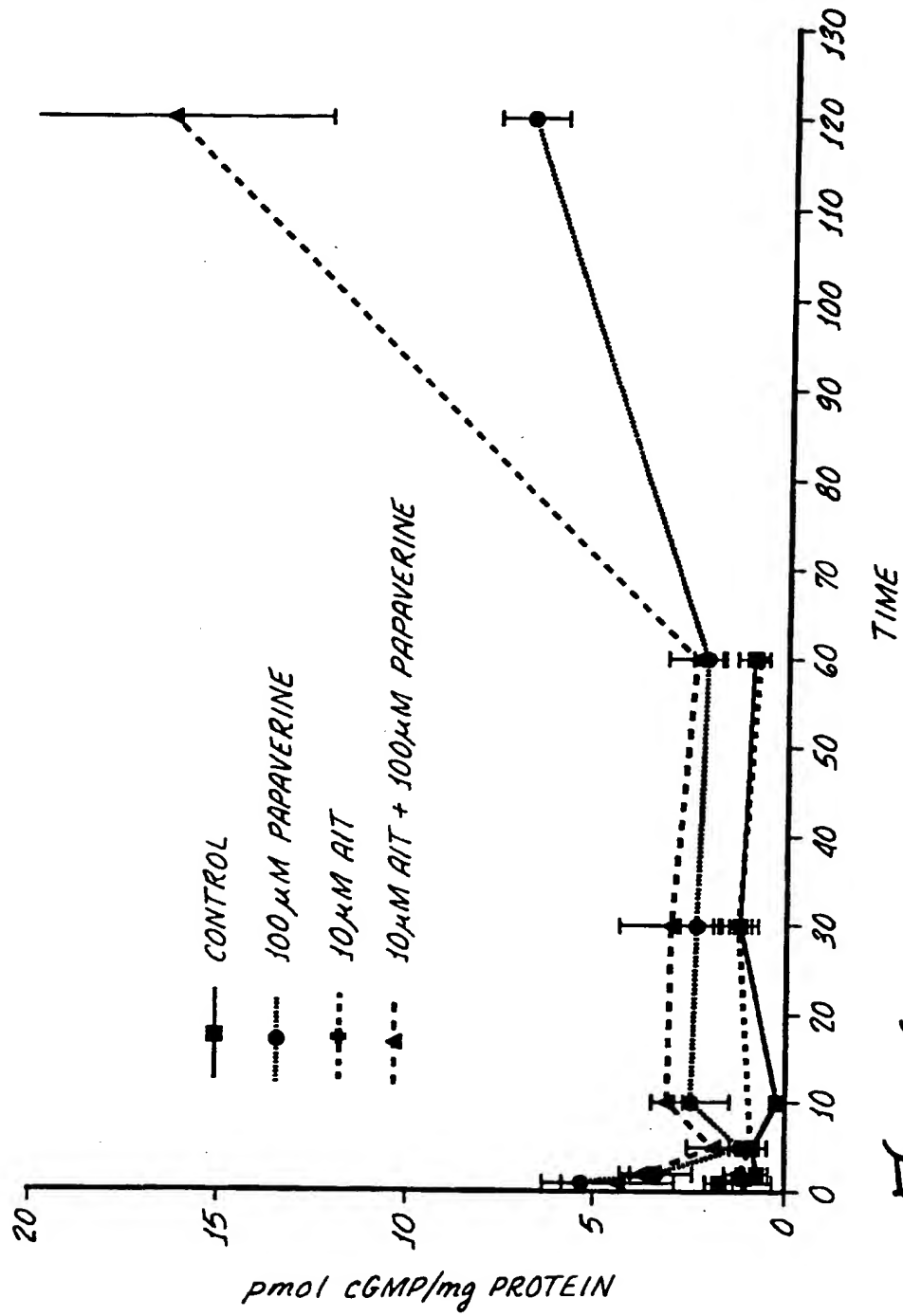


FIG. 6.

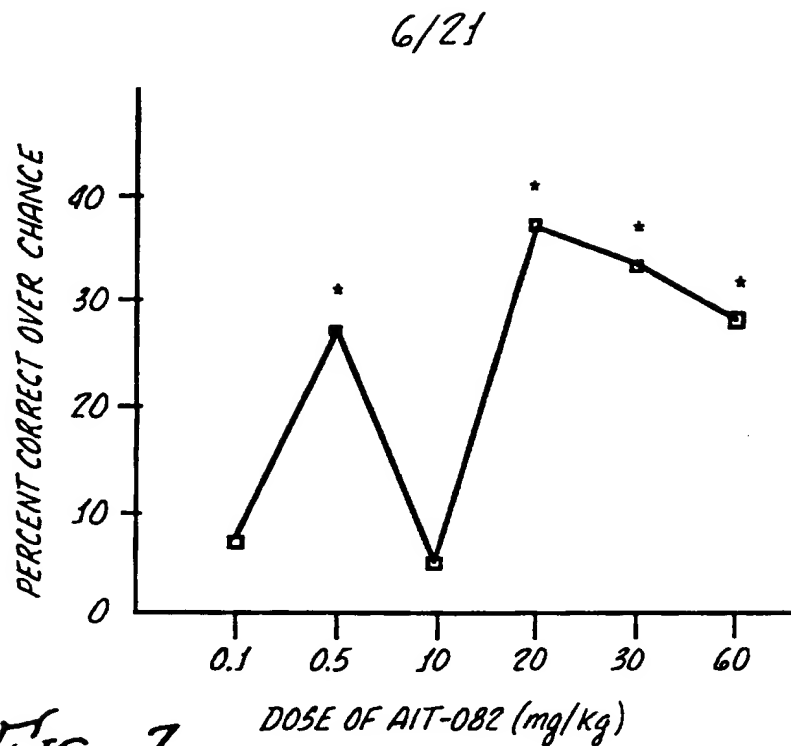


FIG. 7.

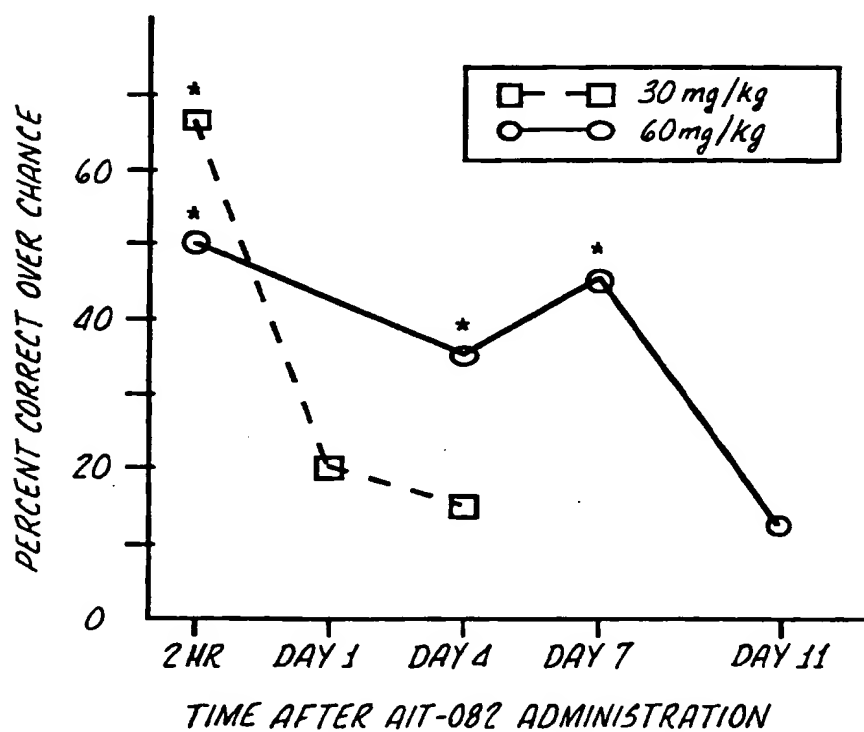


FIG. 8.



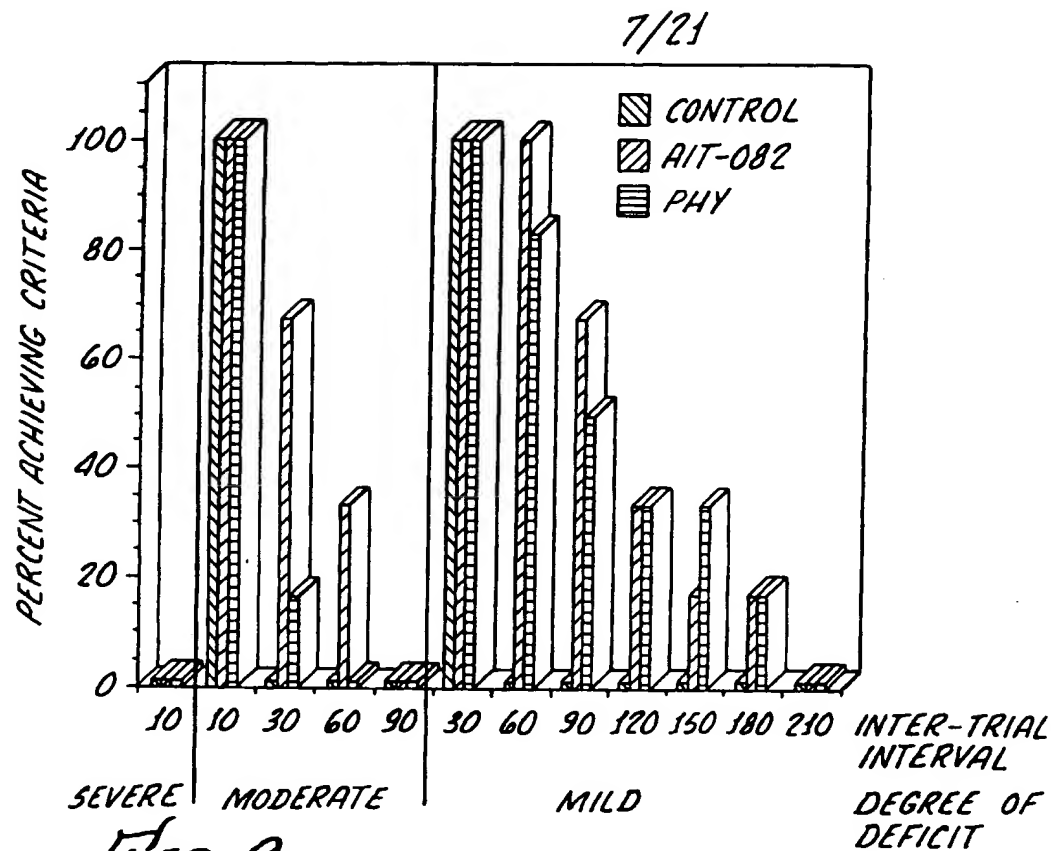


FIG. 9.

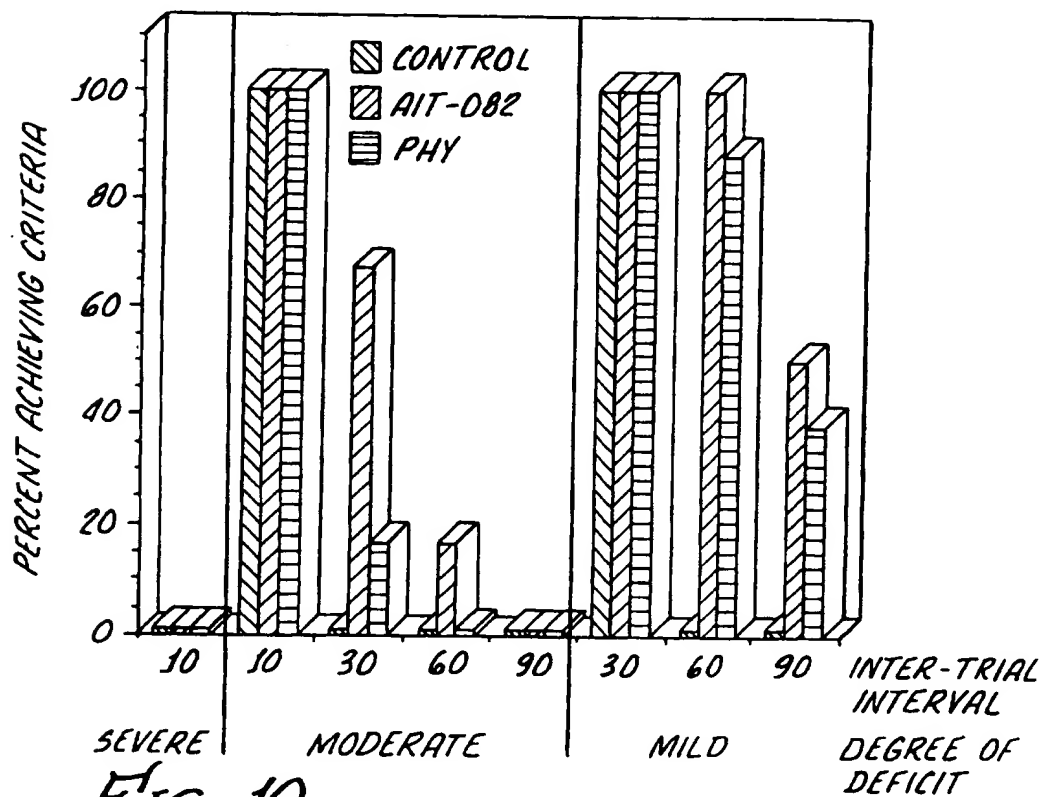


FIG. 10.

8/21

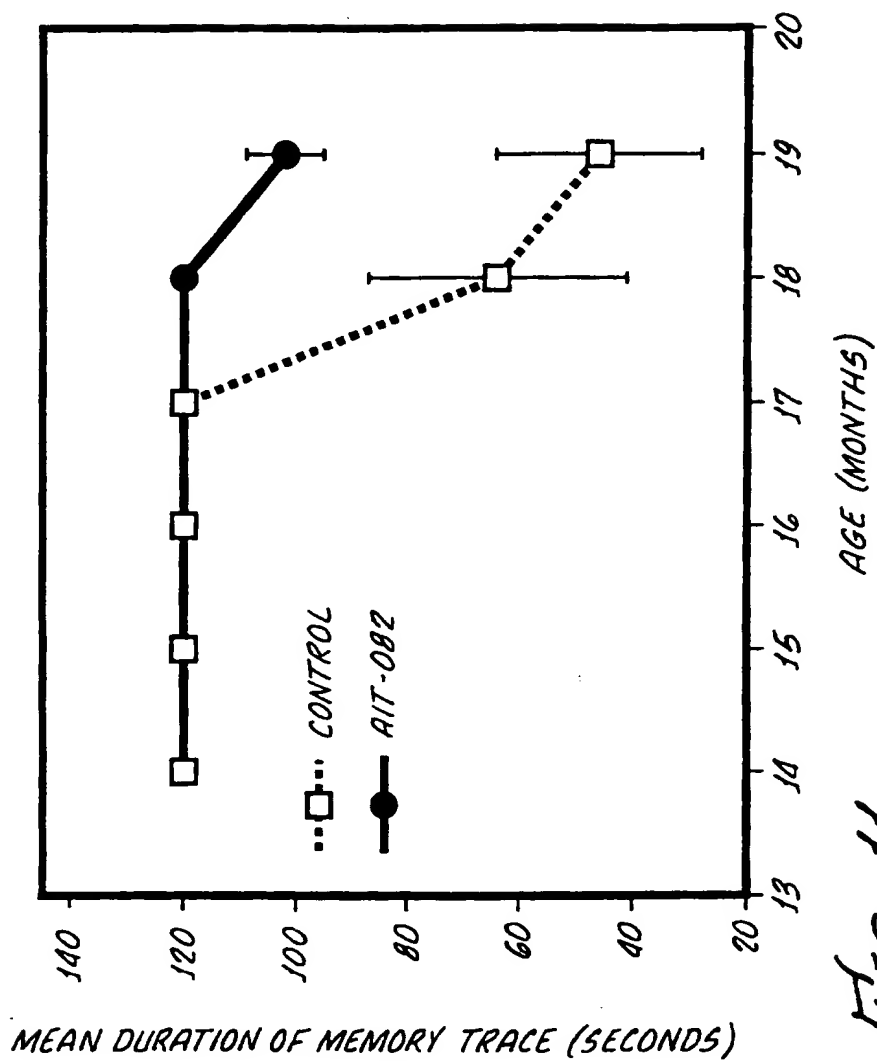
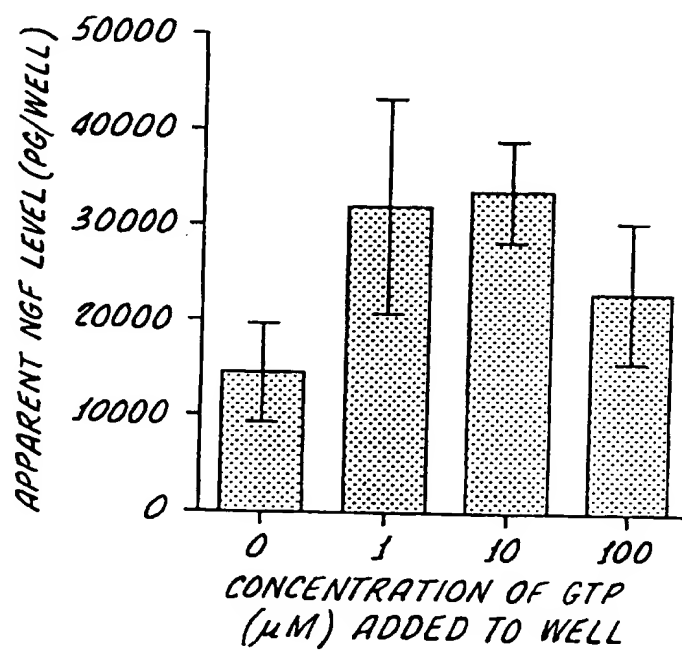
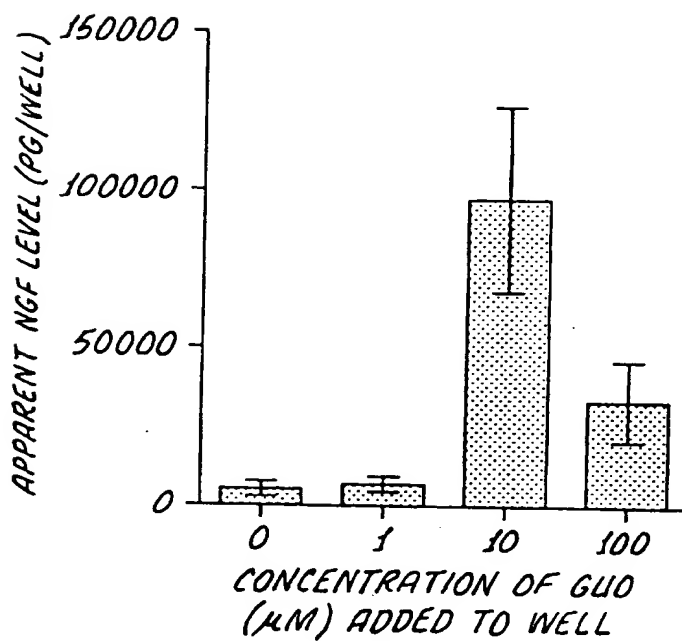
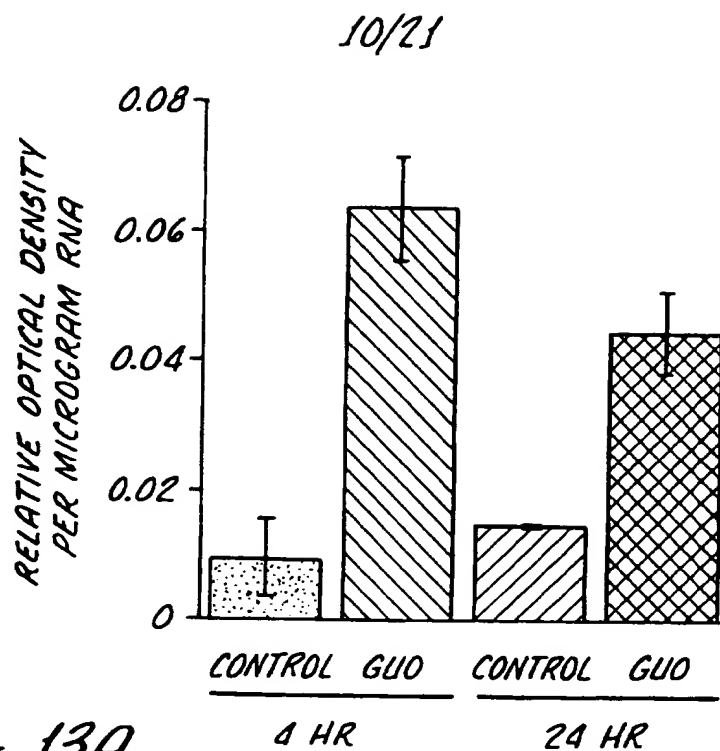
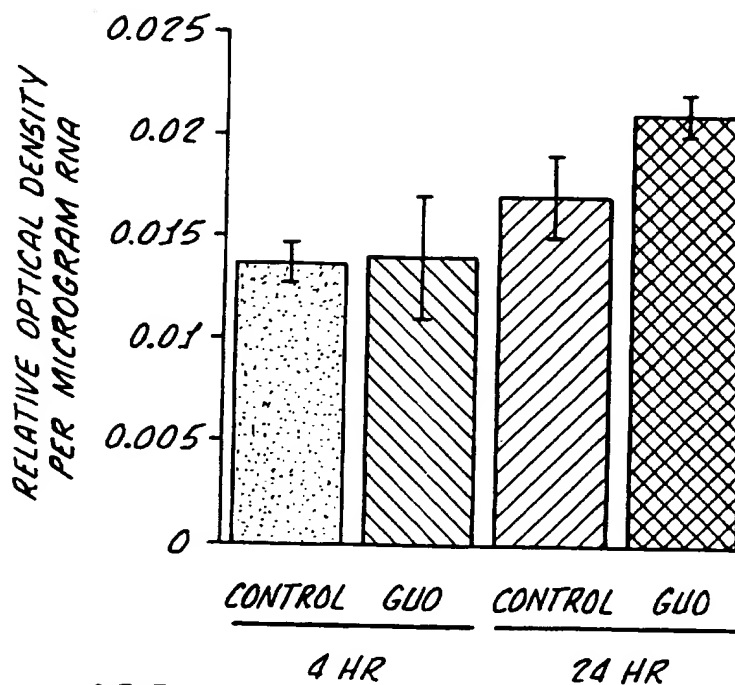


FIG. 11.

9/21

*Fig. 12A.**Fig. 12B.*

FIG. 13A.FIG. 13B.

11/21

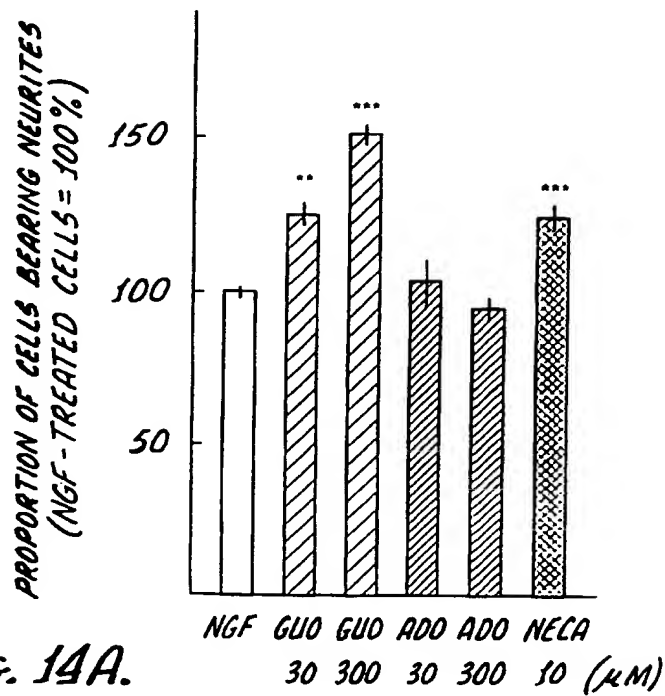


FIG. 14A.

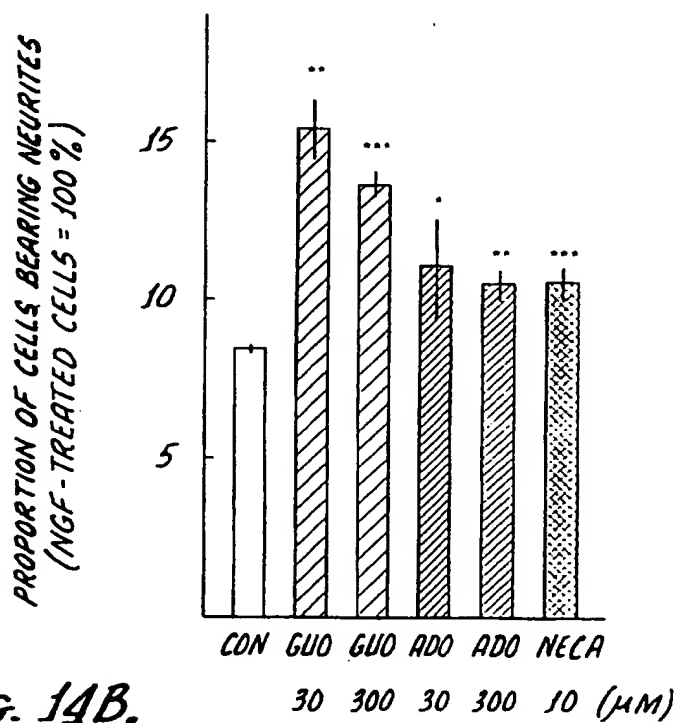


FIG. 14B.

12/21

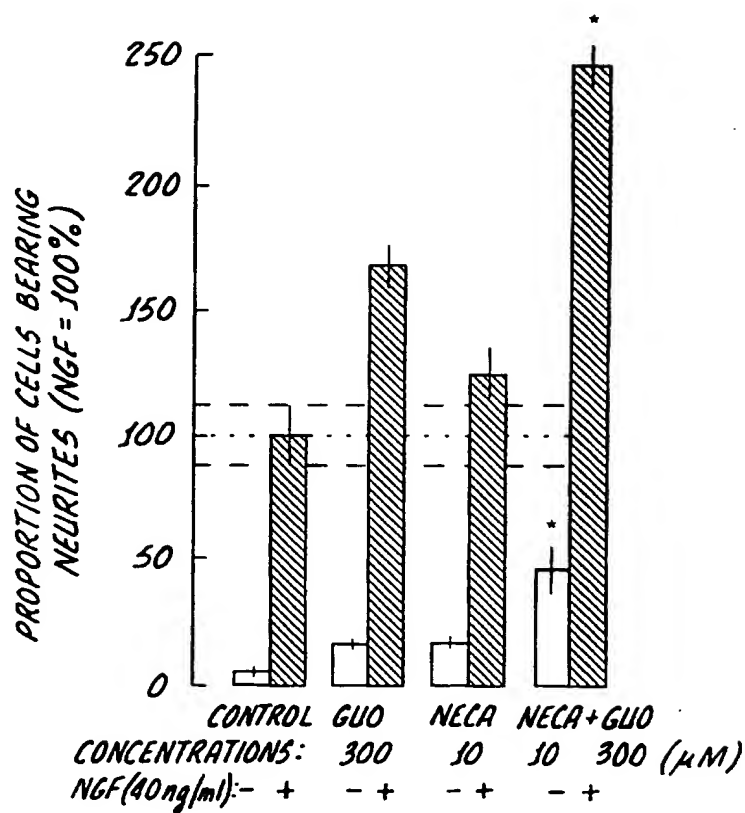


FIG. 14C.

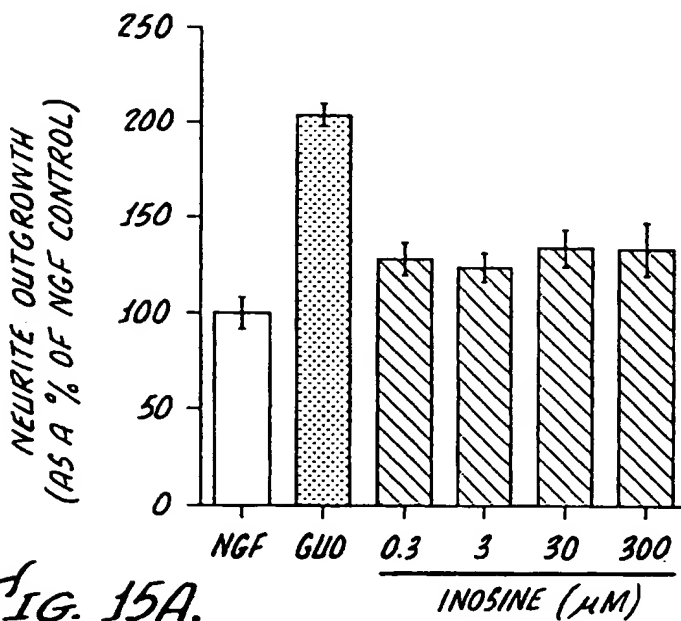


FIG. 15A.

13/21

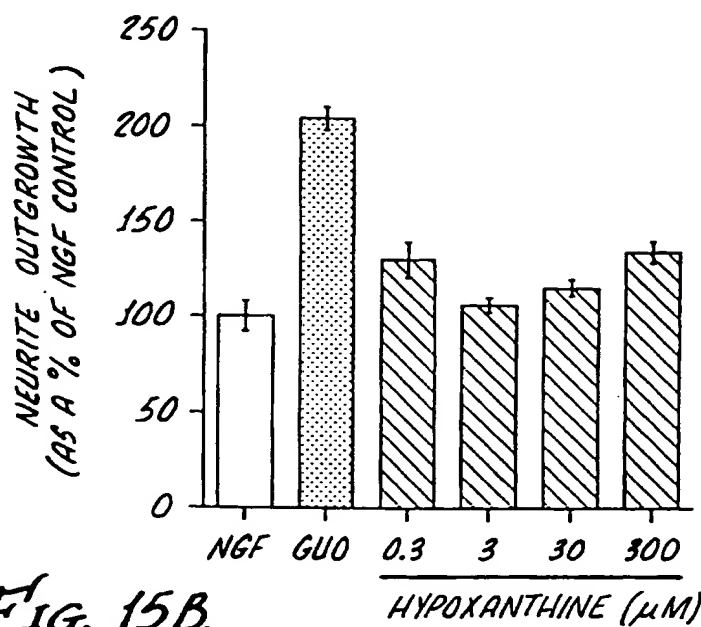


FIG. 15B.

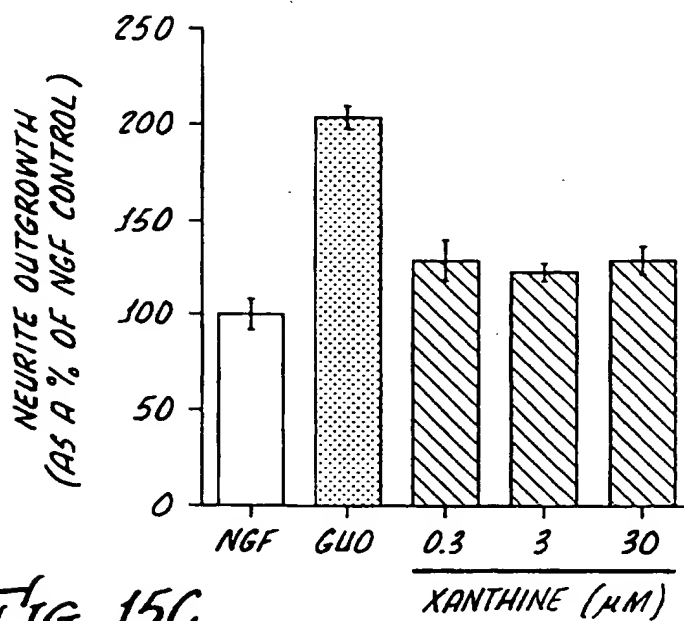


FIG. 15C.

14/21

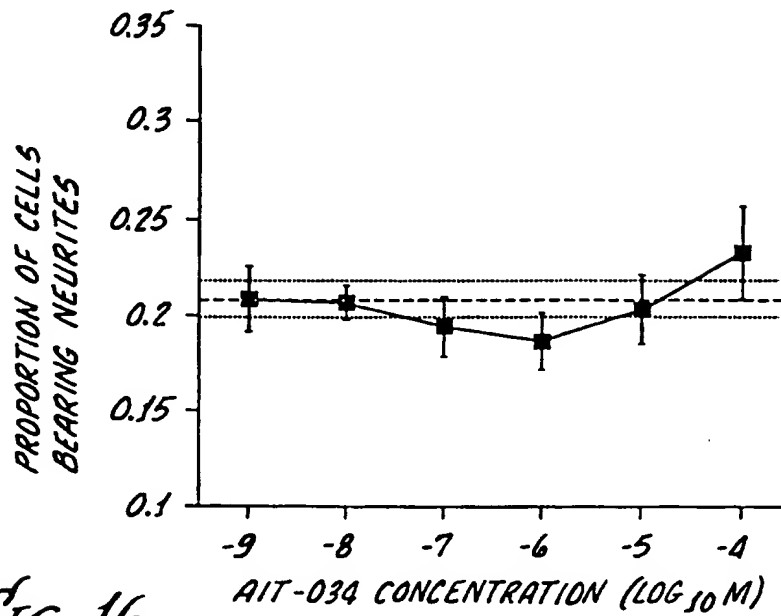


FIG. 16.

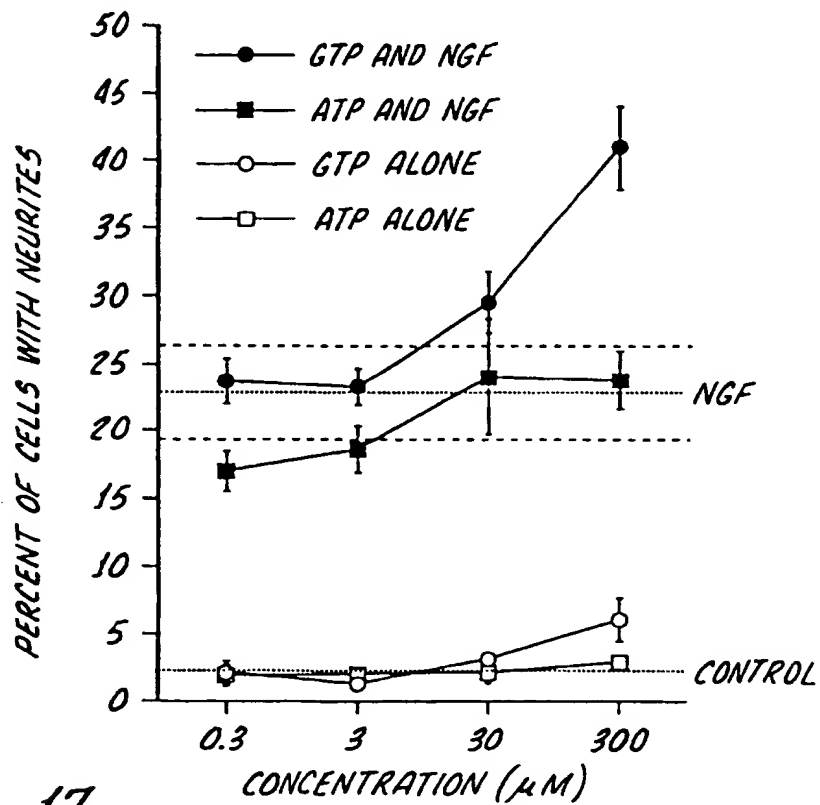


FIG. 17.



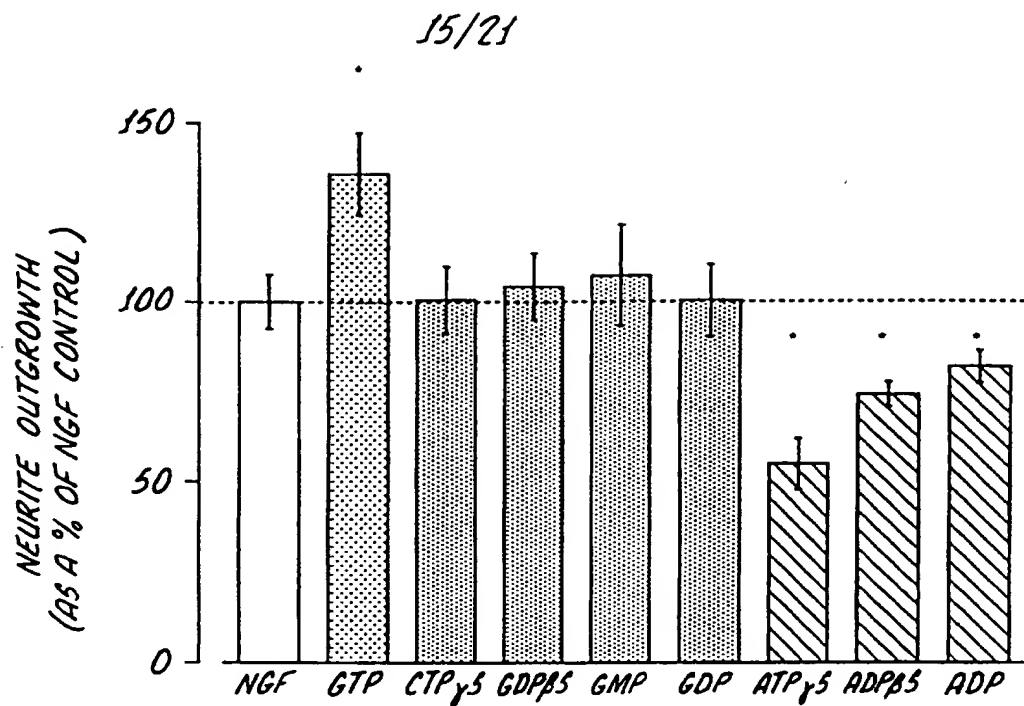


FIG. 18.

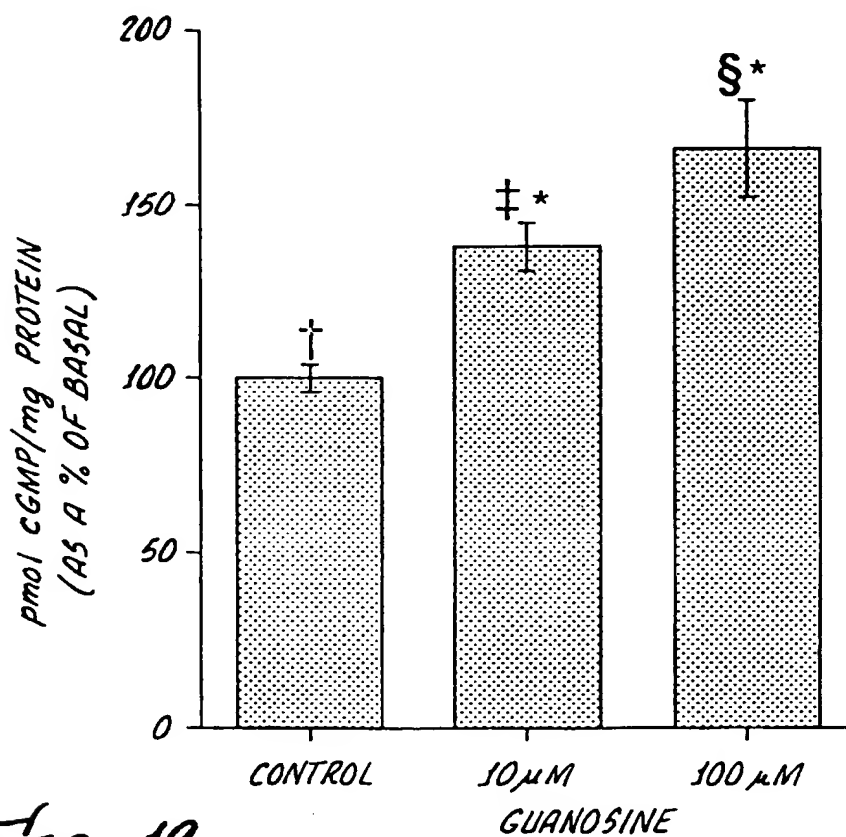


FIG. 19.

16/21

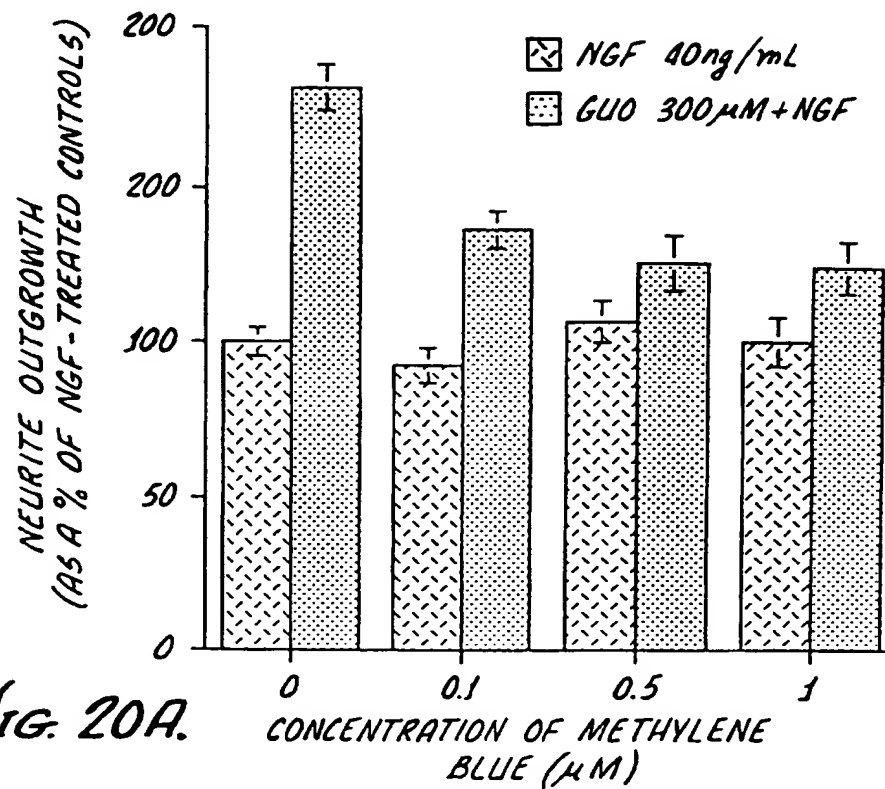


FIG. 20A.

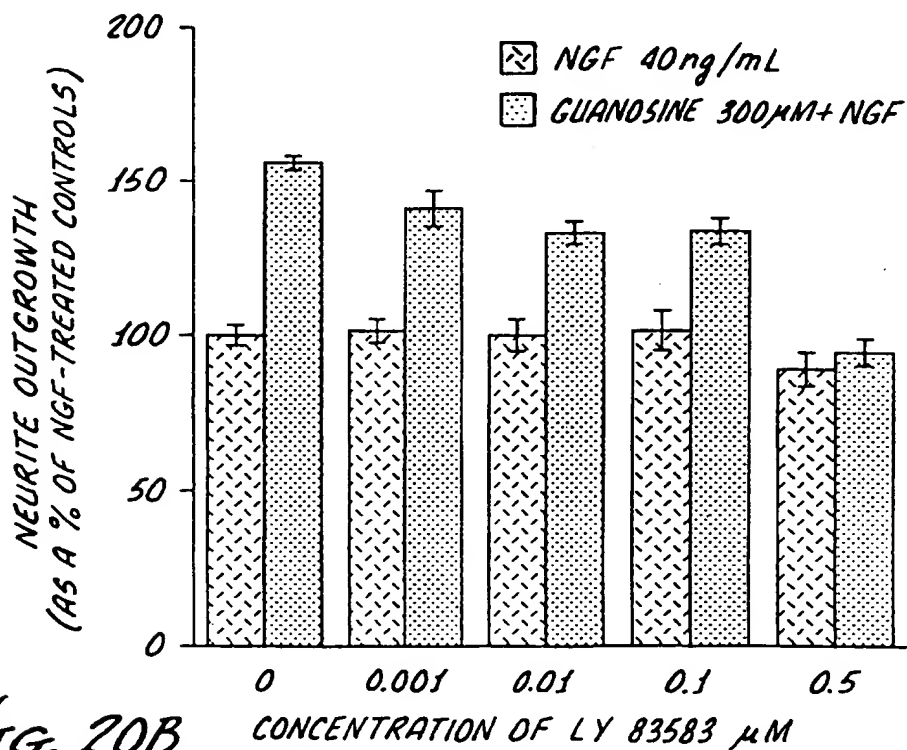


FIG. 20B.

17/21

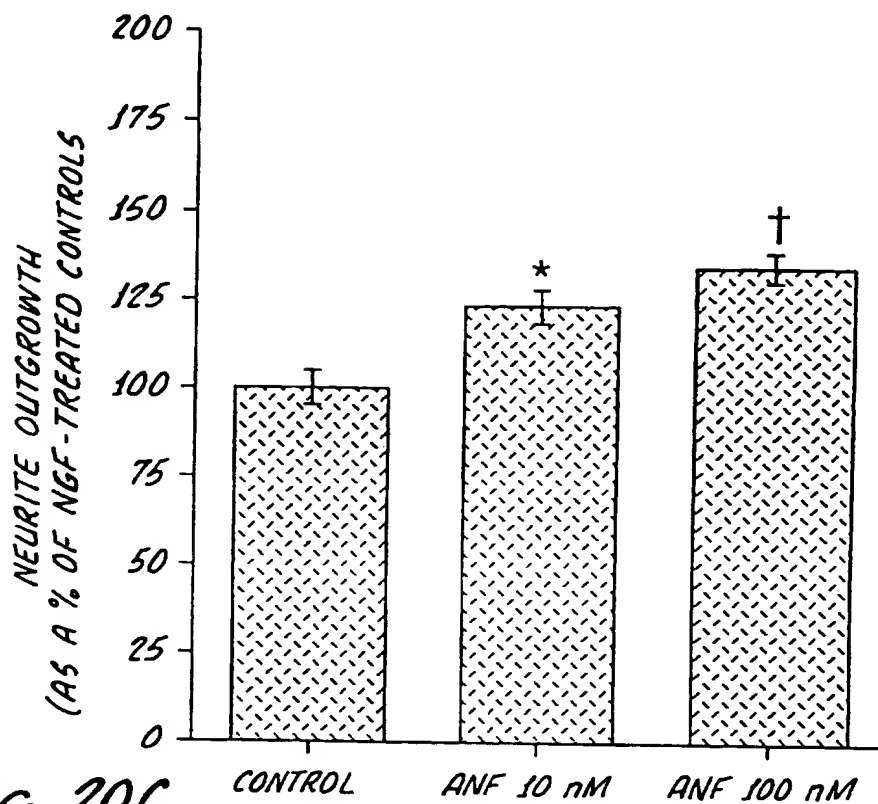


FIG. 20C.

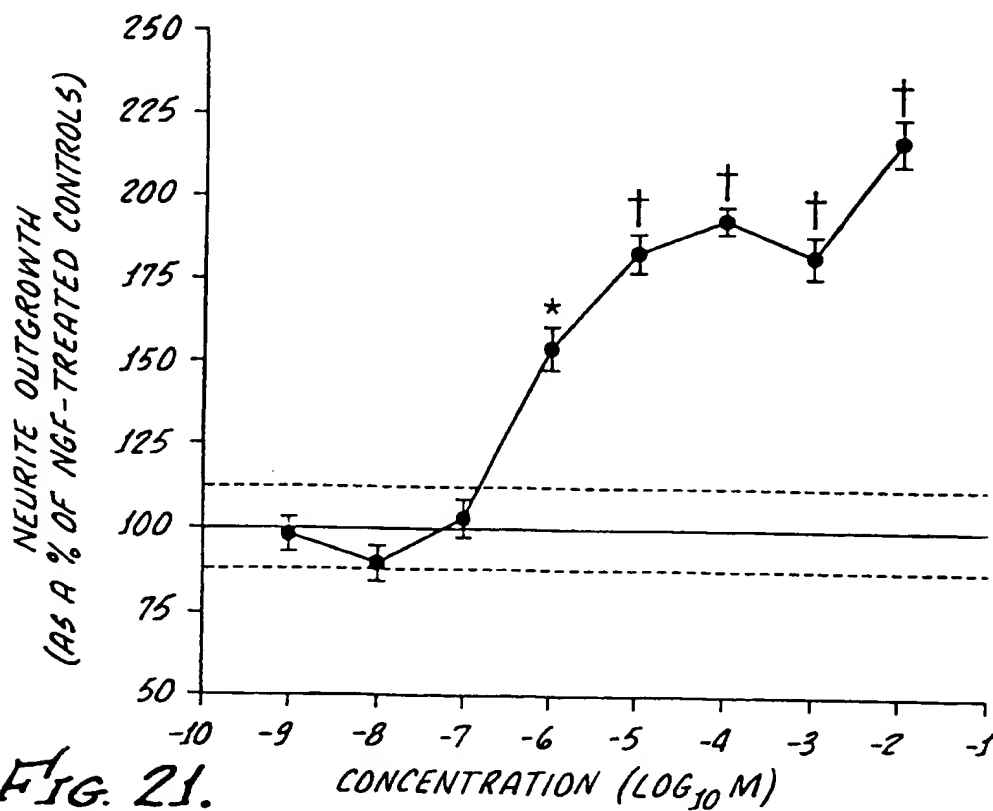


FIG. 21.

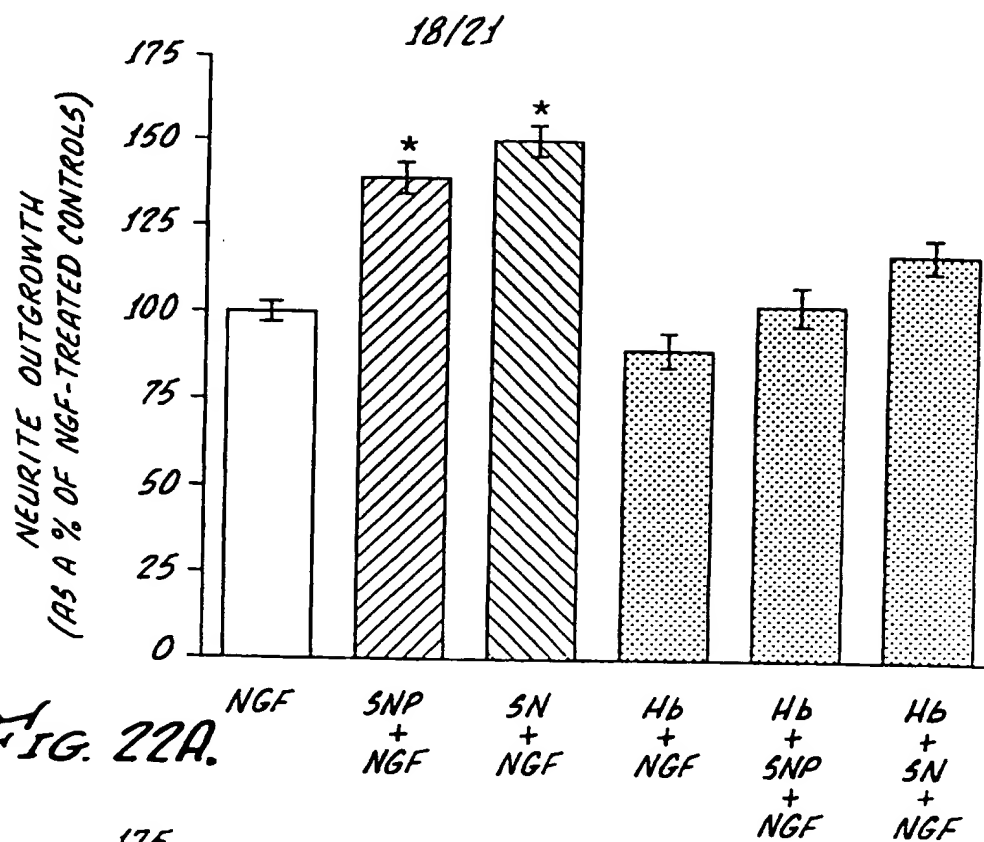


FIG. 22A.

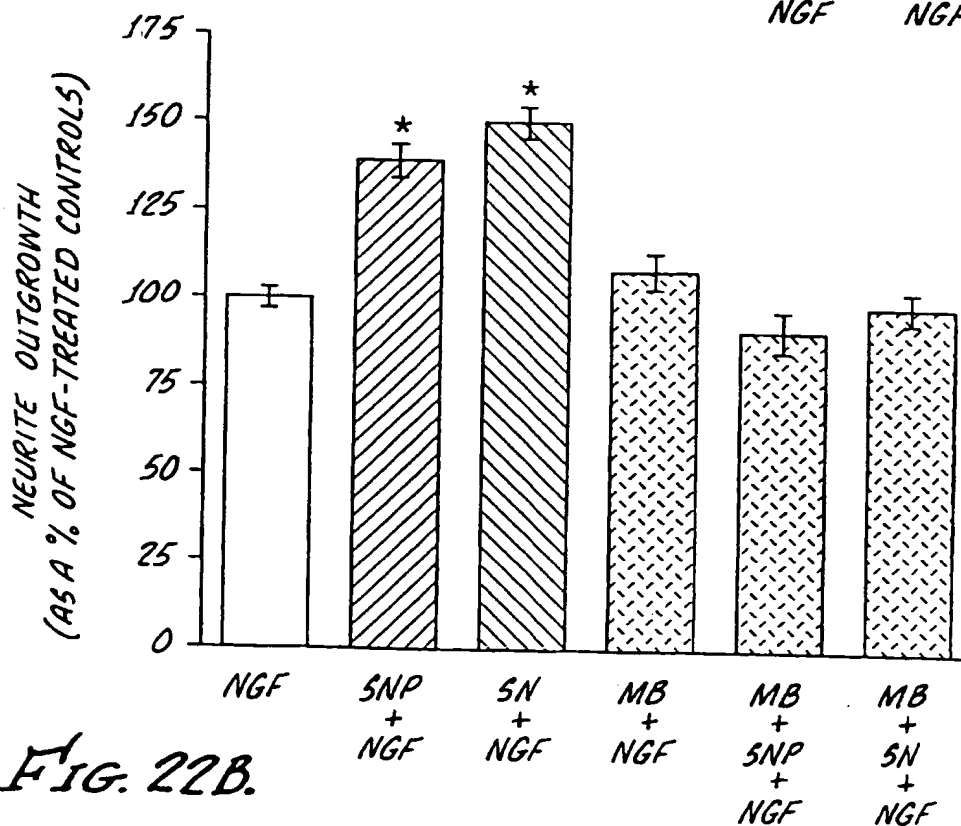


FIG. 22B.

19/21

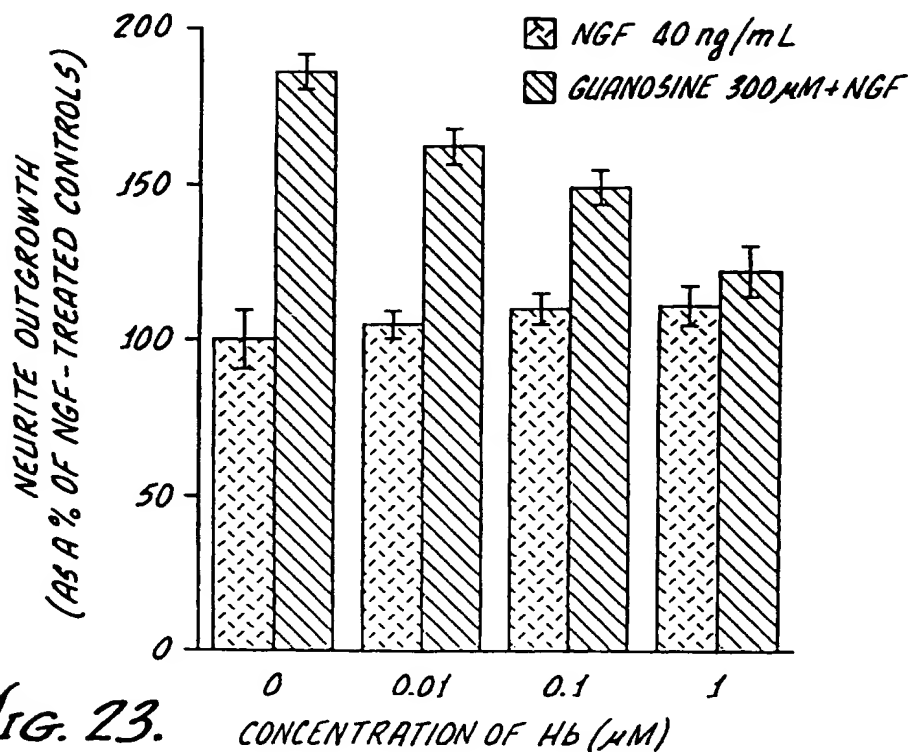


FIG. 23.

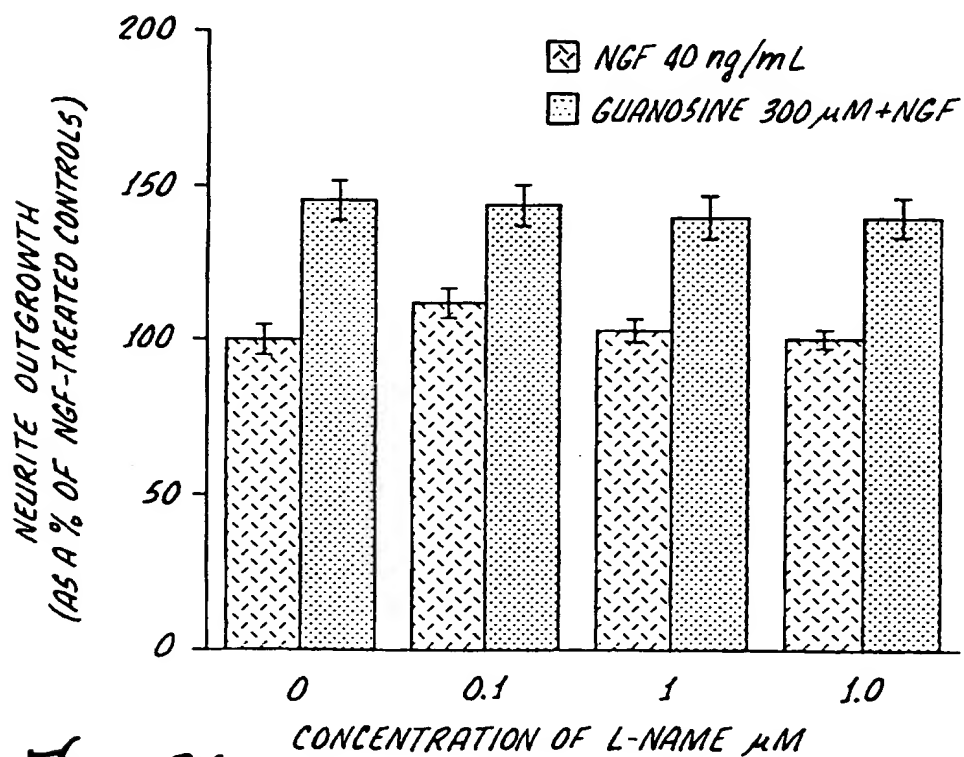


FIG. 24.

20/21

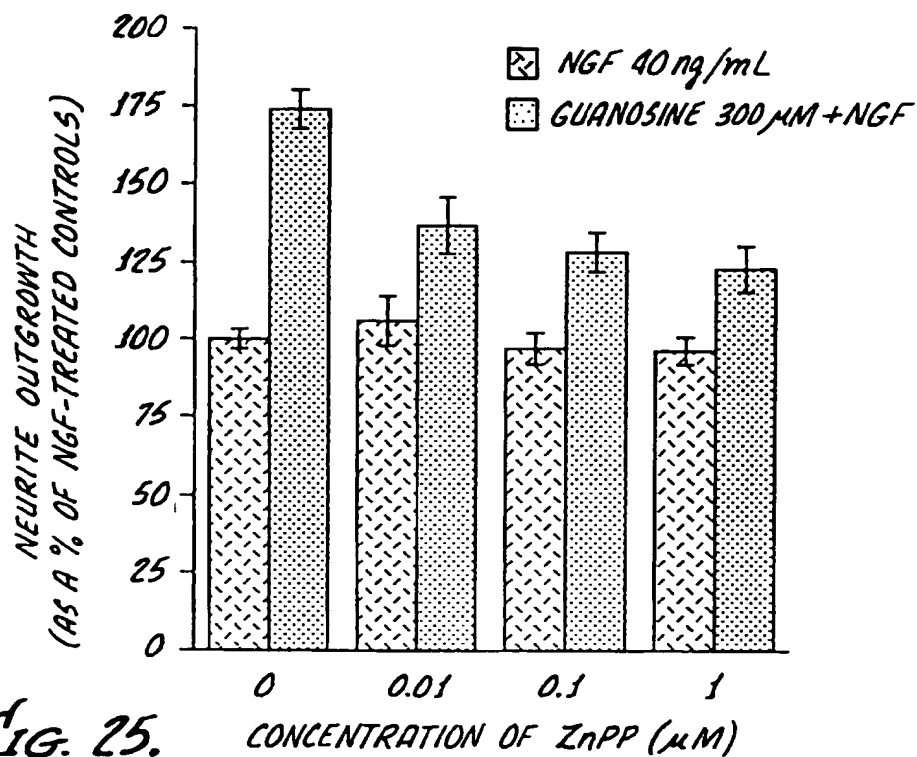


FIG. 25.

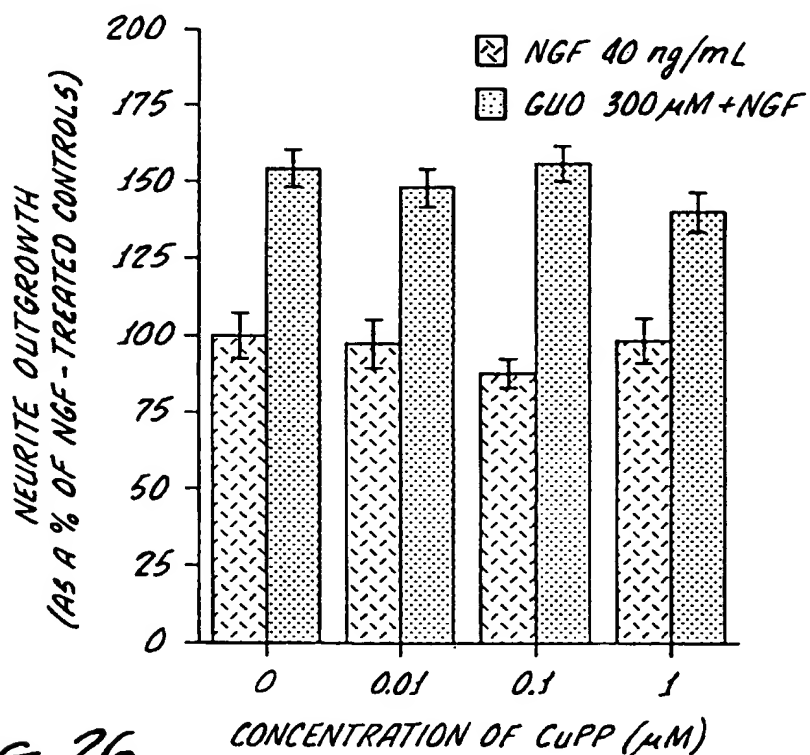
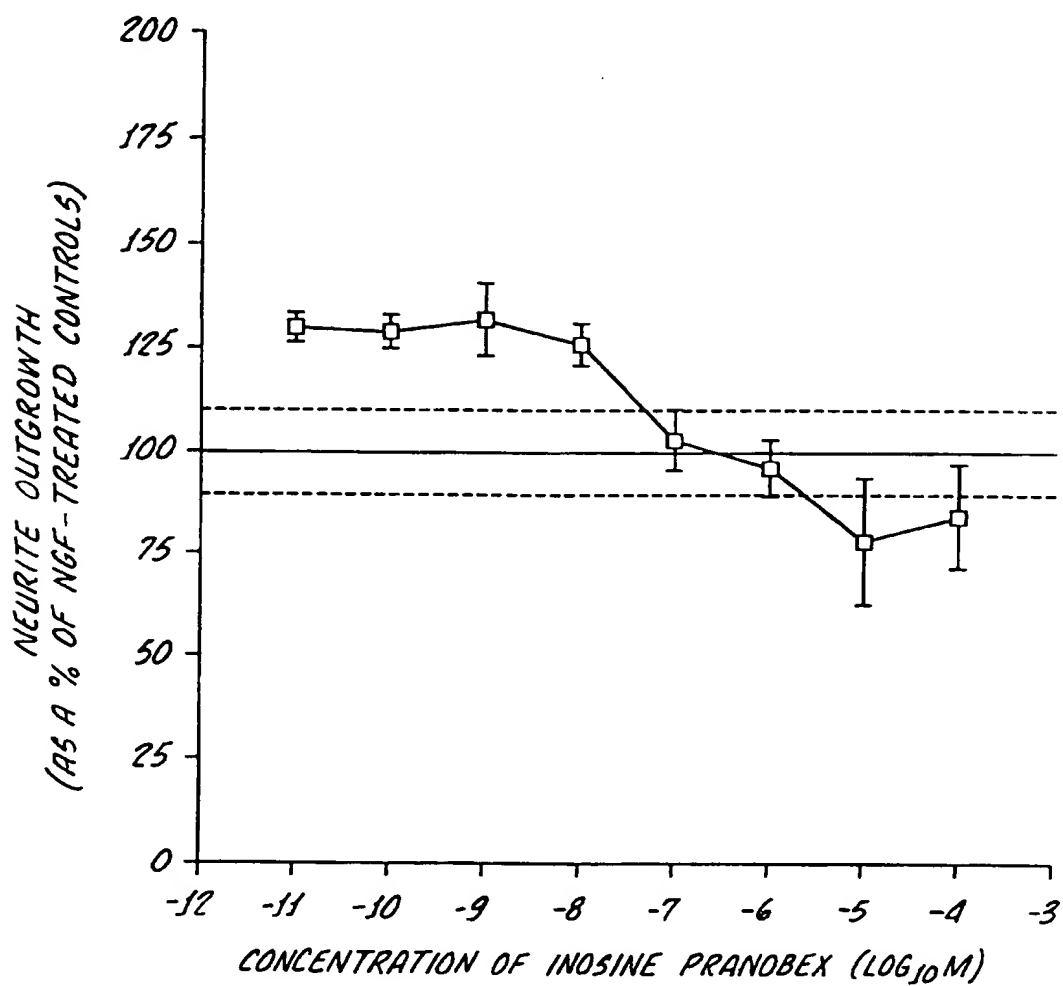


FIG. 26.

21/21

FIG. 27.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/10008

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 31/52; C07D 473/00; C07H 19/167, 19/173

US CL :514/262; 536/27.81; 544/265, 276, 277

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/262; 536/27.81; 544/265, 276, 277

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 4,035,486 (LABORIT) 12 July 1977, column 2, lines 15-33, claim 1.	1-3, 5-17, 19-42, 44-51, 53-60, 62-85, 87-91 ----- 4, 18, 43, 52, 61, 86
X	US, A, 5,091,432 (GLASKY) 25 February 1992, column 4, lines 46-53, example 39, column 34, line 62 to column 35, line 2.	1-91



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 NOVEMBER 1995

Date of mailing of the international search report

06 DEC 1995

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ROSALYND A. WILLIAMS

Telephone No. (703) 308-1235



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/10008

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	US, A, 5,256,677 (SHAM ET AL) 26 October 1993, column 14, line 7 to column 15, line 30.	1, 2, 4-16, 18-38, 40, 41, 43-50, 52-59, 61-84, 86-91 — 3, 17, 39, 42, 51, 60, 85
X — Y	Therapiewoche, Volume 34, No. 17, issued 1984, E. G. Jung, "Chemotherapy of Multiple Warts with Inosine Pranobex (Methisoprinol)", pages 2615-2618, see entire abstract.	1, 2, 4, 6-16, 18, 20-38, 40, 41, 43, 45-50, 52, 54-59, 61, 63-84, 86, 88-91 — 3, 5, 17, 19, 39, 42, 22, 51, 53, 60, 62, 85, 87

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/10008

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STNCAS

search terms: guanosine, 4-3-1,6-dihydro-6-oxo-9H-purin-9-yl-1-oxo-propyl-amino-benzoic acid, inosine pranobex, 36703-88-5, carbon monoxide dependent guanylyl, medicament